Novel Approaches to Assess the Prevalence of Palaeoparasites in European Archaeological Sites



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Thesis submitted for the degree of a
Doctor of Philosophy

Declaration

I, Hannah Frances Ryan, declare that this thesis has never been submitted, either in the same or a different form, to this or any other university, for a degree or any other qualification. All the work presented in this thesis is the result of my own work unless otherwise stated in the author contributions. The work was carried out while I was registered as a graduate student at the Research Laboratory of Archaeology and the History of Art, under the supervision of Prof. Greger Larson and Dr. Adrian L. Smith.

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Abstract

Palaeoparasitology is the study of parasites, in this case helminths, in archaeological contexts such as well-preserved human remains or sedimentary samples. The robust nature of helminth eggs, along with the distinct life cycle of different species has meant that the study of these organisms have provided insight into aspects such as hygiene and subsistence patterns in archaeological sites. However, discussion and research on the health impact of these parasitic organisms in historical populations is lacking from the published literature despite the significance of these organisms for health today. History is a resource to explore the resilience and spread of helminths, nevertheless, to be useful for informing modern practices the analysis of helminths in archaeological sites needs to be robust, quantified and comparable between sites. There were two methodological changes to palaeoparasitology which were investigated in this thesis to address this problem. The first was to produce a proxy for the prevalence rates of helminths in past societies. Most published studies are either undertaken on communal samples, where it is difficult to associate the quantity of helminth to the number of human hosts, or small scale studies of individual burials within a site. In this thesis, a large scale analysis of helminths from single burials in a number of archaeological sites was undertaken, and concordance between prevalence and risk factors in past and present populations was identified. These results were used to explore the effectiveness of different interventions utilised by modern health practitioners. Currently most studies utilise ancient DNA analysis in palaeoparasitology to confirm species identification so, the second goal was to develop genetic methods to produce data that could be used to study population dynamics and dispersal of helminths. This thesis explores the strength and weaknesses of different genetic approaches to palaeoparasitology, which provide a valuable foundation for future studies to identify the most appropriate techniques for their research questions. This thesis shows methods for making robust conclusions on the incidence and factors that control the spread of helminths in historical populations. This is vital if the work is to be of use for informing modern practices.

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Chapter One: Introduction and Literature Review

1.1 Motivation for this thesis

Palaeoparasitology is the study of parasites in archaeological contexts, which was first established in a study by Ruffer (1910), who investigated mummified bodies to study a broad spectrum of pathologies. There is a wide range of parasites that infect humans, as well as the animals they interact with. As these different organisms utilise different routes of infection, they can elucidate broad topics including human-pathogen evolutionary biology, hygiene practices, diet, agriculture and domestication, environmental changes, migration, trade, and more directly, health (Reinhard, 1992). While humans are also infected by bacteria, viruses and ectoparasites, this thesis will focus on helminths. Humans have acquired around 300 different species of helminth worm (Cox, 2002), and a number of these have been recovered from archaeological contexts such as mummies (Ruffer, 1910), bog bodies (Jones, 1986), latrines (Moffet, 1992), middens and pits (Kenward, 2009), environmental samples (Maicher et al., 2017), coprolites (Anastasiou et al., 2012) and single grave burials (Seo et al., 2010).

Despite a vast quantity of research on these organisms, there has been no systematic study investigating the epidemiological significance of them in historical populations. This is in spite of the health impact of these organisms today. The soil transmitted helminths for example, infect more than a billion people which is particularly problematic for children as they can cause malnutrition, growth stunting, intellectual retardation, and cognitive and educational deficits (Bethony et al., 2006). This lack of research may be partially down to the focus of studies on species identification in archaeological contexts.

The majority of microscopic studies are either on communal samples, or small-scale studies of individuals, which makes it impossible to quantify the prevalence of helminths in a way that is epidemiologically significant (De Rouffignac, 1987). In other words, we cannot trace the incidence, routes of infection and factors that control the spread of helminths in historical populations. Even ancient DNA techniques which have been successfully used to trace the spread and evolution of pathogenic organisms such as *Yersinia pestis* (Bos et al., 2011; Spyrou et al., 2018) and *Mycobacterium tuberculosis* (Bos et al., 2014; Stucki et al., 2016), have been underemployed and predominantly limited to species identification for the study of helminths (Côté and Le Bailly, 2017). This results in diametric data, presence or absence, of the different helminths in an archaeological site. Whilst this data can show which populations suffered from helminth infections, it cannot be used to indicate the degree to which these pathogens were a

problem. Therefore to remain accurate, most statements of the health impact of helminths in historical populations are broad.

Studies of palaeoepidemiology provides novel case examples to explore how different ways of life impacted the prevalence and risk of pathogenic organisms (Milner and Boldsen, 2017). This thesis aims to start a narrative on the methods that can be applied for the study of palaeoparasitology so that specific, quantified statements of the spread, rise, and fall of helminths in historical populations can be made. By building a clear picture of the significance of helminths in the archaeological record, we can begin to explore the mechanisms by which these helminth populations became a global phenomenon and were maintained from prehistory, through to modern populations. This can be done by transitioning the study away from solely species identification, to an approach where helminths are quantified by microscopy, and population dynamics are modelled using ancient DNA analysis. Studies such as these have the potential to provide insight that can be used by modern medical practitioners or policy makers. As there is a broad spectrum of helminth taxa, the obvious place to begin is to focus on the helminths that are most commonly identified in the archaeological record.

1.2 What are helminths?

Helminth is not a taxonomic unit, but instead a catch all name for parasitic worms. Species that are considered as helminths come from three phyla: *Platyhelminthes* (flukes and tapeworms), *Nematoda* (roundworm) and *Acanthocephala* (thorny-headed worm). In archaeology, these parasites are found in a wide range of contexts. Helminths that infect any region of the human body can be detected in mummies and bog bodies however, these context types are rare in the archaeological record. Sedimentary samples on the other hand, are much more readily recovered and these sample types, along with coprolites, can contain gastrointestinal helminth eggs which are released into the environment with faecal waste. As this thesis is focused on producing wide-scale epidemiological data for historical populations, sedimentary samples and gastrointestinal helminths are the main focus of this research.

The most notable helminths for our research are two of the soil transmitted helminths (STH), the nematodes *Ascaris* spp. and *Trichuris trichiura* (*T. trichiura*). They are two of the most common helminths to infect modern populations and two of the most common helminths found in archaeological contexts (Araújo et al., 2015). In this thesis the cestodes *Diphyllobothrium latum* and *Taenia* spp. were also recovered during microscopic analysis and *Macracanthorhynchus* sp. was the focus of analysis on museum collections. The life cycle and biology of these helminths is summarised here

to highlight the difference that the recovery of these helminths means for inferring human behaviour. The biological information below is taken from the CDC DPDx (CDC 2019) resource and the description of the morphology of the eggs comes from the WHO's Bench Aid for the diagnosis of intestinal parasites (WHO, 1994).

1.2.1 *Ascaris* spp.

Ascaris spp. are large worms, with the adult females ranging between 20-35 cm and the males between 15-30 cm. As adults they are found in the lumen of the small intestines of humans. Adult worms live between 1-2 years with a female producing around 200,000 eggs per day. When fertile, *Ascaris* spp. eggs are oval in shape and measure 55-75 μm by 35-50 μm with mamillations on its surface. When the eggs are infertile, they are elongated and measure 85-95 μm by 43-47 μm, lack structure and have an irregular mamillated layer. Once expelled, fertile eggs take from 18 days to several weeks to become infective. If the eggs are ingested whilst infective, the larvae hatch and invade the mucosa before eventually being carried to the lungs where they mature. After 10-14 days they make their way to the throat, are swallowed and end up back in the small intestines. The life cycle is summarised in Figure 1.1a.

Ascaris lumbricoides (A. lumbricoides) is usually described as the human-specific *Ascaris* but, there is some debate whether it is actually the same species as *Ascaris suum* (*A. suum*) which used to be perceived as a pig specific species (Betson and Stothard, 2016; da Silva Alves et al., 2016; Leles et al., 2012; Peng and Criscione, 2012; Shao et al., 2014; Søe et al., 2016). This differentiation was supported by the fact that the adult *Ascaris* spp. worms found in humans were morphologically distinct from those found in pigs (Leles et al., 2012). The capacity for cross infection (Nejsum et al., 2012) combined with the genetic similarity of the two species (Leles et al., 2012) brings this distinction into question. For this thesis the distinction between the two is not addressed and therefore eggs identified will be referred to as *Ascaris* spp. as a conservative approach.

1.2.2 *Trichuris trichiura*

T. trichiura are smaller than *Ascaris* spp. measuring about 4 cm in length. They are also unique in their morphology, hence why they are colloquially known as whipworm. As adults, the worms are fixed in the cecum and ascending colon with their anterior portions threaded into the mucosa. They live as adults for about a year and the females release between 3,000 to 20,000 eggs per day. *T. trichiura* eggs are smooth and rugby ball in shape with bipolar plugs, measuring 50-55 μm by 22-24 μm. Un-embryonated eggs pass with stool and become infective in the environment after 10-15

days. Once ingested the eggs hatch in the small intestines before settling in the ascending colon. The life cycle is summarised in Figure 1.1b. There are *Trichuris* species that can cross infect into humans (such as *Trichuris suis* see, Nejsum et al., 2012) however there is no debate as to whether these are the same species.

1.2.3 *Taenia* spp.

Two species of *Taenia* are commonly found in humans, *Taenia saginata* (*T. saginata* or beef tapeworm) and *Taenia solium* (*T. solium* or pork tapeworm). Both of these enter humans by the consumption of under cooked, under cured, or raw red meat. The species of *Taenia* will be indicative of whether the individual was eating infected pork or beef. The adults can be found in humans in the small intestine attached by their scolex. *T. saginata* worms are usually 5 m or less (but can reach 25 m long) and *T. solium* adults are usually between 2-7 m. Rather than releasing eggs in the host, adults produce proglottids that become gravid and are passed with the stool. These proglottids release the eggs after they are passed with the faeces and each proglottid may contain 50,000 (*T. solium*) or 100,000 (*T. saginata*) eggs. These two species of *Taenia* have virtually identical eggs that are 31-43 μm in diameter with a thick prismatic shell wall. Once the eggs are released from the human host they can stay infective for months in the environment. Cattle or pigs become infected by ingesting eggs from the environment, then the oncospheres invade the intestinal walls and migrate to the striated muscle, where they develop into cysticerci. These can survive for several years in the animal host. On consumption of contaminated meat, humans become infected. The life cycle is summarised in Figure 1.1c.

1.2.4 *Diphyllobothrium latum*

Like *Taenia* spp., *D. latum* have intermediate hosts. *D. latum* enter humans through the consumption of under cooked, under cured or raw freshwater fish. As adults they are located in the small intestines of humans attached to the humans' intestinal mucosa by the bilateral groves (bothria) of their scolex. Adults can reach more than 10 m in length and up to 1,000,000 eggs can be released per day per worm. The eggs are operculated and measure 58-75 μm by 40-50 μm. There can be a small protuberance at the abopercular end but in ancient samples this is unlikely to be recovered. Once the eggs are released into the environment via human faeces, they mature and develop into a coracidia. These are then ingested by a suitable freshwater crustacean where they develop into procercoid larvae. When the crustacean is consumed by a secondary intermediate host (often minnows and small freshwater fish), the procercoid larvae are released from the crustacean and migrate into the secondary hosts flesh where they

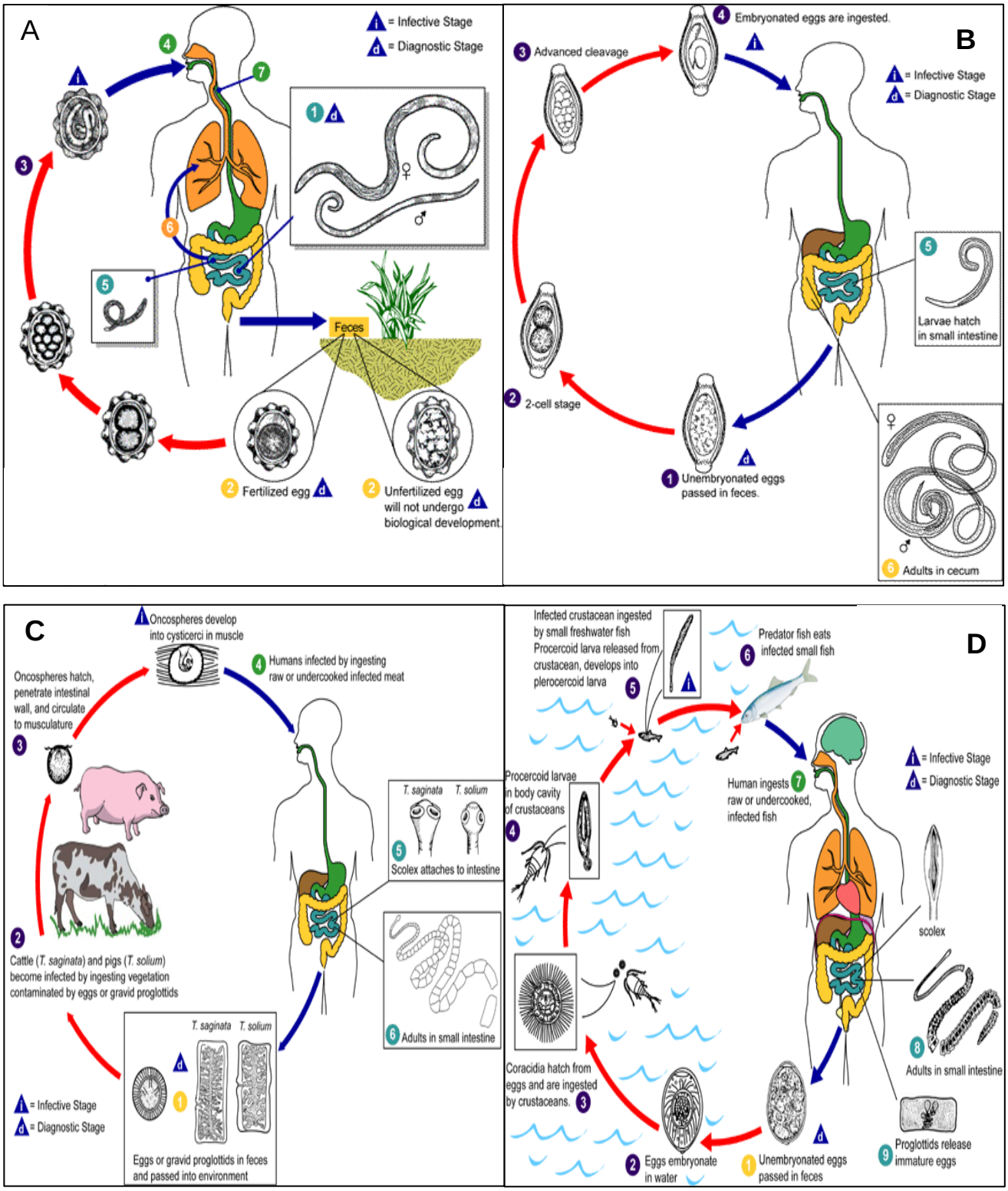


Figure 1.1: Life Cycle of A) Ascaris spp. B) T. trichiura C) Taenia spp. and D) D. latum. Images are all sourced from the Centre for Disease Control (CDC 2019). Reference does not constitute its endorsement or recommendation by the U.S. Government, Department of Health and Human Services, or Centers for Disease Control and Prevention. Images are available on the agency website for no charge.

develop into a plerocercoid larvae. At this stage the larvae are infective in humans but usually the plerocercoid larvae are passed up the food chain and humans become infected by eating larger predator fish. The life cycle is summarised in Figure 1.1d.

1.2.5 *Macracanthorhynchus* spp.

Macracanthorhynchus spp. are not specialised to infect humans, but there are cases of accidental infection. The definitive hosts are: rats for *M. moniliformis,* racoons for *M. ingens* and swine for *M. hirudinaceus*. The eggs are shed with the definitive hosts' faeces which are then ingested by an intermediate insect host, usually scarabaeoid or hydrophilid beetles for *M. hirudinaceus* and beetles or cockroaches for *M. moniliformis* and *M. ingens*, where they develop into a second larval stage. The definitive host becomes infected after the consumption of the intermediate host. The life cycle is summarised in Figure 1.2. The eggs are 80-100 μm long by 50 μm wide, ovoid in shape with a thick textured shell. Whilst infection is rare in humans, they can cause symptoms like abdominal pain, fever, nausea, and vomiting.

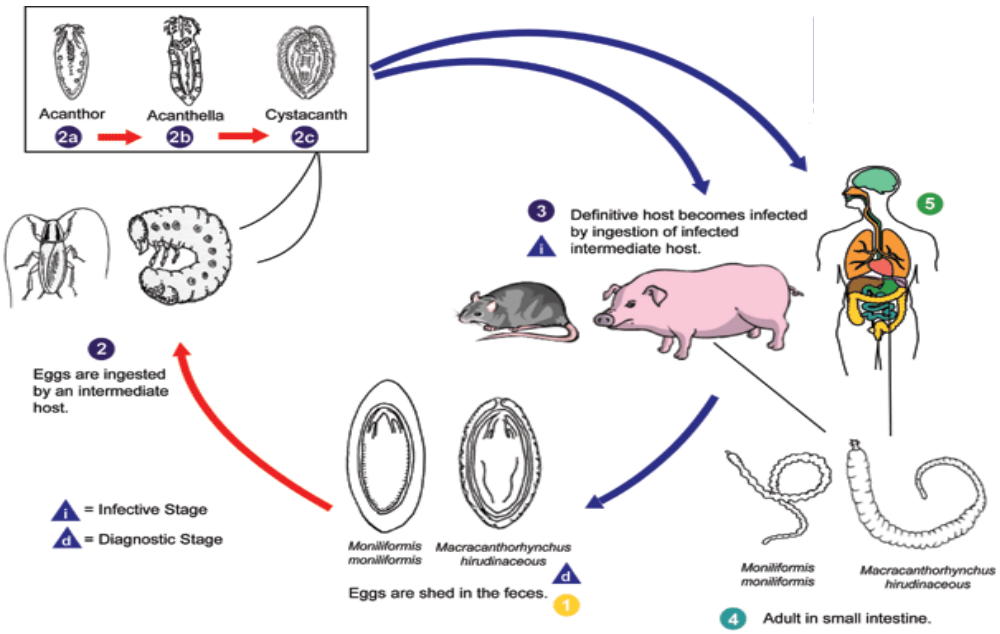


Figure 1.2: Life Cycle of Macracanthorhynchus spp. Image sourced from the Centre for Disease Control (CDC 2019). Reference does not constitute its endorsement or recommendation by the U.S. Government, Department of Health and Human Services, or Centers for Disease Control and Prevention. Images are available on the agency website for no charge.

1.3 Pathology – the significance of helminths today

Four out of five of the genera of helminth studied in this thesis have humans as their definitive host (*Ascaris* spp. *T. trichiura*, *Taenia* spp. and *D. latum*). These helminth are therefore specialized to infect humans and consequently are endemic infections in modern human populations today. The prevalence and pathology associated with these species does however differ. *Ascaris* spp. and *T. trichiura* are the two that are the most

medically significant. The World Health Organization (WHO) has classified STH (including *Ascaris* spp. and *T. trichiura*) as one of the 17 top neglected tropical diseases (Hunt et al., 2016). In 2010 it was estimated that 819 million people were infected with *Ascaris* spp. and 464.6 million were infected with *T. trichiura* (Pullan et al., 2014). These helminths can cause obstruction, anorexia, mucosal damage and irritation leading to malabsorption in the gastrointestinal tract which can result in growth retardation in children (Stephenson et al., 2000). The morbidity of these helminths disproportionately effects those living in poverty. They preferentially proliferate in the environments that stem from poverty, and contribute to the poverty cycle by lowering productivity due to loss of manpower, impairment of childhood education, and additional treatment costs (Gazzinelli et al., 2012).

In 2001 WHO endorsed a resolution (WHA54.19) urging endemic countries to start seriously tackling worms (WHO 2017). Since then, wide scale chemotherapy of School Aged Children (SAC) has been the main tool of choice and the proportion of children who are heavily infected by soil transmitted helminths is now less than 1% (Becker et al., 2018). As can be seen in Figure 1.3, a high number of children still require treatment to control the morbidity effects of STH, and they tend to be concentrated in resource poor countries. Whilst the WHO resolution may act to control some of the pathology associated with these helminths in children, many argue that this resolution will have limited effect in eradicating these species (Anderson et al., 2013; Ásbjörnsdóttir et al., 2017; Truscott et al., 2014). This is due to the fact that adults are an effective refuge for helminth reinfection into the community, as is the contaminated environment (Bopda et al., 2016; Truscott et al., 2014). Helminth infection in adults is usually mild or asymptomatic therefore, this group are a lower priority for treatment.

Much of the literature is focused on STH however, there is a growing recognition that cysticercosis, caused by *T. solium*, is a major health issue that is growing in parallel to increasing pig production (Montresor and Palmer, 2006). *T. solium* was added by WHO to the list of major neglected tropical diseases in 2010 but there is still a sparsity of reliable epidemiological data for this species (WHO, 2015). It is of note that the endemicity of *T. solium,* as can be seen in Figure 1.4, has a high degree of overlap with the geographic spread of soil transmitted helminthiases.

Medical cases of *T. saginata* are reported globally, but the most robust data on its distribution comes from slaughterhouses where prevalence rates have been found to be up to 6.8% in some European countries (Webb and Cabada, 2017). The lack of more robust data may be due to the fact that *T. saginata* does not cause cysticercosis in

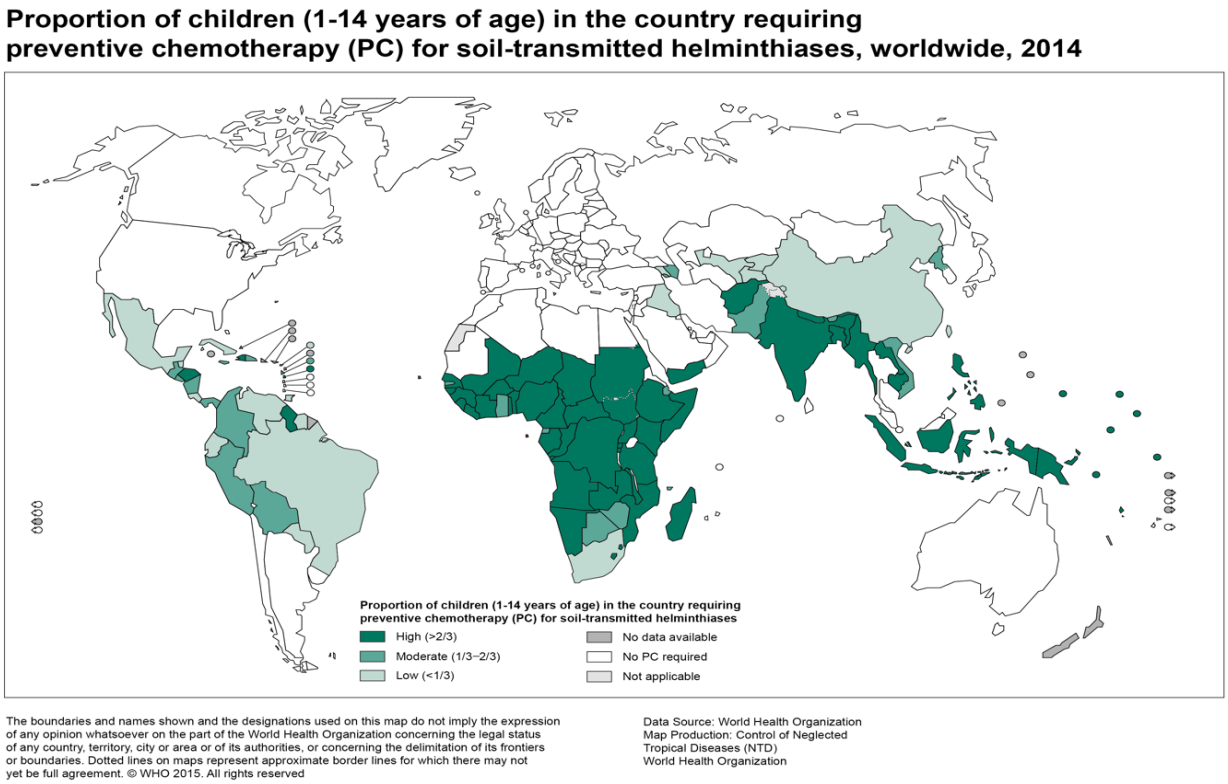


Figure 1.3: Number of children (PRE-SAC and SAC) requiring preventive chemotherapy for soil transmitted helminthiases. Image sourced from WHO 2014

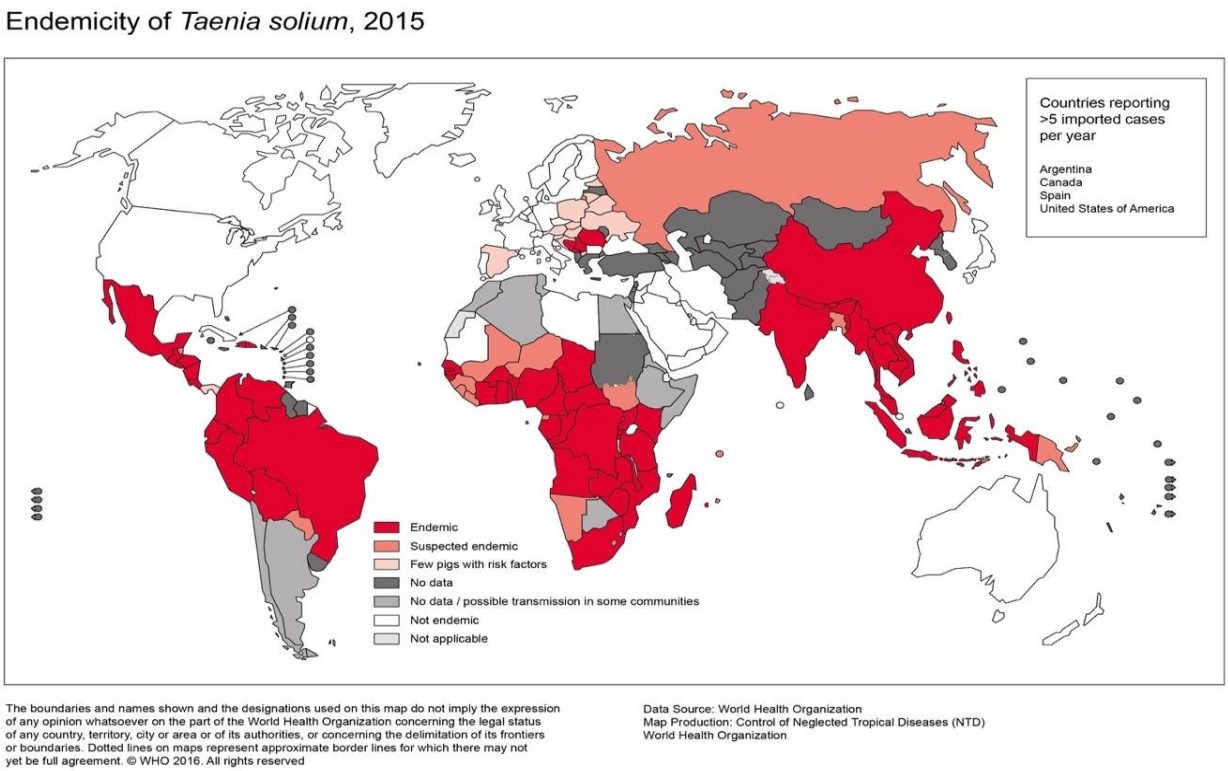


Figure 1.4: A map of endemicity of Taenia solium. Image sourced from WHO 2015

humans, unlike *T. solium*. Diphyllobothriosis, caused by *Diphyllobothrium* spp., on the other hand is reported to be on the decline in North America, Asia and most of Europe, but on the rise in Russia, South Korea, Japan and South America (Scholz et al., 2009). Despite occurring widely in the world, the reports of these cases are fragmentary as it is considered to be a mild disease. Both *T. saginata* and *D. latum* do not have the same impact on the health of modern populations and therefore there is not the same level of detailed medical research on these species.

1.4 Historical Helminths

As well as being modern global phenomena, the helminths described above have also been prolific in historical populations. Helminths have been described in ancient texts such as Egyptian papyri, Hippocrates medical texts (460 to 375 BC), and script from Roman physicians including Celsus (25 BC to AD 50) and Galen (Galenus of Pergamon, AD 129 to 200) (Cox, 2002). Not only this, they have regularly been identified from archaeological sites throughout history right back to the prehistoric period (Bouchet et al., 2003). Unlike other infective bodies, helminths are predominantly studied by the recovery and identification of their eggs by light microscopy. These eggs have characteristics that make them particularly well adapted for study in the archaeological record.

1.4.1 The survival of and identification of helminth eggs

The study of helminths is fruitful for archaeologists due to the robust nature of helminth eggs and the specific and obligate nature of the helminths' life cycle. The robust nature of helminth eggs comes from their necessity to survive outside a host organism so the species can proliferate. This may mean an egg is exposed to the environment for long periods of time depending on where it is deposited and whether the host species is migratory and/or is solitary or social. Whilst different classes of helminth (*Digenea*, *Cestoda*, *Acanthocephala* and *Nematoda*) have different structures and compositions to their eggs, they all have multiple layers with different functions to protect the embryo within (Wharton, 1983).

This strong structure means that a range of helminth taxa can survive sewage treatment processes (Johnson et al., 1998; Storey, 1987) and some chemical or household disinfectants (El-Dakhly et al., 2018; Gadelhaq et al., 2018; Khueangchiangkhwang et al., 2018; Oh et al., 2016). Nematodes in particular have a very robust egg shell structure including an inner lipid layer, middle chitinous layer and outer vitelline layer, with covalent linkage between the chitin and protein providing additional resistance to chemical attack (Florenzano et al., 2012; Wharton, 1980). The

robust nature of helminth eggs allows the structure to remain intact and visually identifiable using light microscopy.

Fortunately, the morphology of the eggs alone can be used to distinguish between helminth taxa. Many genera can be readily distinguished by light microscopy but, differentiating species can be more difficult. Even if only the genera can be discerned, this can still be used to identify parts of the life cycle to explore a broad spectrum of human behaviours. The main differences in the life cycles, as discussed above, include how the helminths are passed between organisms (ingestion of contaminated food, water ways or via the faecal-oral route) or the number and specificity of host organisms. The identification of a wide number of parasites within an archaeological context can provide a holistic understanding of factors such as health, subsistence and hygiene. Despite the significance of helminth to health in modern populations, the latter two factors are usually the focus of palaeoparasitological studies.

1.4.2 Interpretation of helminths to highlight subsistence practices

In archaeological sites, the intermittent hosts of human helminths can be used to indicate what humans were eating, and the definitive host of non-human helminths can indicate which animals were in close proximity to the human population (Reinhard et al., 2013). These can both be used to indicate subsistence practices. One example where a shift in dietary practice was traced by a change in the helminth population is by Flammer et al. (2018). In the medieval European site of Lübeck, there was a distinct temporal shift from *D. latum* being predominantly recovered in communal latrines to *T. saginata*. The rise in ova of *T. saginata*, along with faunal remains, showed a cultural change from the consumption of fish to the consumption of beef. As Lübeck was a trading port, these changes may reflect changing relationships within Europe and the importance of different commodities for trade. Alternatively, the rise of leather work along the river may have made the fish inedible or reduced the number of copepods, which are indicators of water quality, and this disrupted the completion of the life cycle of *D. latum*. In addition to this change in subsistence, the presence of these helminths was indicative of how the food was prepared. Either intentionally or accidentally, food must have been regularly consumed raw or undercooked/cured.

As well as tracing diet directly, helminth eggs can also be used to trace the timing of domestication and the species that were domesticated (Morand, 2012). For example, in northern Chile the presence *Enterobius macusaniensis* in archaeological contexts indicated the exploitation of llamas which were used for food, transport and sacrificial offerings (de Souza et al., 2018). A shift towards agriculture more generally, theoretically

leads to increased parasitic transmission from: proximity to livestock, higher insect densities, consistent water sources, increased contact with faeces due to permanent settlements and grain storage leading to greater contact with rodents (Betson et al., 2014; Perry, 2014). A rise in parasitism coincided with agriculture in Europe however, in many parts of the Americas the higher reliance on wild animals and lower population concentration during the agricultural revolution mitigated the risk of parasite infection (Reinhard et al., 2013). This highlights that the relationship between behavior and parasitism is not always direct. For example, agriculture is strongly associated with diet, but as it can also increase contact with faeces, hygiene and sanitation practices can mitigate or amplify the increased risk associated with agriculture.

1.4.3 Interpretation of helminths to highlight hygiene practices

Sanitation, hygiene and waste management are all practices that can affect human's exposure to pathogenic organisms, including helminths. The cestodes *Taenia* spp. and *D. latum* for example can be spread by poor food hygiene practices. Cooking or curing food thoroughly, kills any bacteria or cestode larvae present and stops the transmission of these infectious bodies. Other species of helminth, such as the nematodes *Ascaris* spp. and *T. trichiura*, are spread via the faecal-oral pathway and therefore proliferate well in societies with poor access to hygiene facilities and education. These two helminths are consequently strong indicators of the local average sanitation levels in historical populations.

Palaeoparasitology cannot be used to identify the types of sanitary practices, whereas written sources, artifacts (sponges, bath tubs etc.) and archaeological structures (latrines, viaducts, bath houses etc.) can. Instead, palaeoparasitology is complimentary as it can be used to indicate the effectiveness of these behaviours for interrupting the faecal-oral routes of infection. Sanitation covers a broad range of behaviours and infrastructures but, not all of these are effective against the spread of pathogenic organisms, particularly when used in isolation (Ásbjörnsdóttir et al., 2017). Despite the Romans having a reputation for cleanliness due to their construction of public washing facilities, sewer systems and piped drinking water, Mitchell (2016) found in a review of archaeological sites that helminths were identified across the Roman empire. This includes helminths spread by the faecal-oral route of infection. The endemic nature of these helminths suggests that the Roman hygiene practices were either not effective at disrupting the faecal-oral route of infection, or, mitigated by other practices that increased risk of infection such as unclean communal facilities or the use of human faeces as fertiliser.

As well as indicating the effectiveness of hygiene practices, helminths can also be used to trace changes to these behaviours. In Newport, Rhode Island, between the 17-20th centuries, changes to the dominant helminth type was used to infer changes in waste management practices that were made to cope with the demands of increasing urbanization (Trigg et al., 2017). Meanwhile, differences in helminth prevalence and type within a community could indicate individual differences in hygiene practice. Fisher et al. (2007) assessed the content of latrines dating between the 18-19th in conjunction with historical records from Albany, New York, to identify whether homesteads of different 'social status' had different levels of sanitation. They found a low prevalence of roundworm in two homesteads representing wealthy and educated families however, the authors suggested that rather than a difference in hygiene, this reflected medical treatment as the prevalence of *T. trichiura* in these households was equivalent to other locations within the city. Hygiene practices have a clear relationship to the health of past populations. While the body of work discussing the association of helminths with hygiene would suggest that there has been an equivalent degree of discussion around helminths and health, in reality it is not considered in the same level of detail.

1.5 Visual Identification of past pathology

Good sanitation reduces the transmission of diseases, particularly those that are transmitted from faeces to new hosts. Despite the clear impact helminths have in modern populations, discussion of their impact on health in historical populations is usually limited to a side-note in research focusing on hygiene practices. Some authors argue that health inferences of helminths in the past are tenuous as their morbidity is dependent on other factors such as: the general nutritional status of the host population, other infectious organisms in the host population and, the immune status of infected individuals (Reinhard, 1992). There is always a degree of uncertainty of the morbidity effects of diseases and infections in historical populations however, other pathologies are regularly studied in the archaeological record regardless. Rather than focusing on morbidity, historical pathology studies instead focus on identifying and quantifying the presence of these infective bodies instead.

1.5.1 Quantifying pathology in archaeology

Disease prevalence and population health is a large area of research in archaeology as it informs researchers of a range of factors that affected these historical populations, be it nutrition, sanitation, predominant work roles or urbanization (Milner and Boldsen, 2017). Many pathologies are traced through osteological examination but this approach is limited in a number of ways. For an illness to be traced in the archaeological record by osteology it has to: either directly affect the skeleton or occur

in a region adjacent to bone, be severe enough to be visible after taphonomic degradation and even when a disease can be traced through osteological examination, skeletal tissue only has limited responses to disease (White et al., 2011). Despite these limitations, the frequency of skeletal pathologies are reported and there been a recent shift in the community towards epidemiological approaches to these datasets (Milner and Boldsen, 2017).

Currently, the frequency of helminths in humans is rarely reported in the archaeological record. This may reflect the type of contexts that are predominantly studied. Sedimentary samples are more readily accessible than bog bodies, mummies or coprolites and therefore are more regularly examined. These samples can either be recovered from communal contexts (such as latrines and waste pits) or single graves. In paleoparasitology, utilising the former is more appealing due to the higher concentration of eggs, and therefore higher likelihood of producing a positive result. There are nevertheless, other limitations.

1.5.2 Impact of archaeological context on quantifying helminths

A single communal context can provide a numerical assessment (eggs per gram) of the parasites present at the site. There are however, some problems with the analysis and comparison of the frequency of helminths between communal contexts. The first is that not all published studies quantify the eggs that are present in a site. For some published reports the identification of ova is used purely as a tool to discern which contexts were used for human waste, so only the presence or absence of helminth ova is reported. A second issue is that it is nearly impossible to ascertain the number of people that contributed to the helminth population in a communal deposit. Jones (1985) quantified the number of worms required for the number of ova detected in archaeological sites but unfortunately, the number of worms does not necessarily have a linear relationship to the number of hosts. Finally, as De Rouffignac states of communal samples:

“Quantitative data are of little value, however, because of factors such as dilution of sewage, biodegradation and erratic egg production by the worms” (1987).

If the quantitative values are ignored and only the diametric data of presence and absence is used to assess prevalence, this would require the assumption that all archaeological contexts had an equal chance of containing ova. This is clearly not the case. For example, enclosed latrines are more likely than pits to have helminths within them, and even different types of latrine will affect the probability of recovering helminth evidence (Fisher et al., 2007). This also limits the ability to compare helminths that are

systematically found, such as *Ascaris* spp. and *T. trichiura,* as they only become notable when absent.

The alternative is single burial analysis. Due to the low success rate when studying single graves for palaeoparasitology, most studies are either limited to a single individual or a small collection of graves. Notable exceptions include Seo et al. (2010) who carried out an analysis of 75 burials to assess parasite recovery in relation to preservation, and Anastasiou et al. (2018) who studied 25 burials to assess infection from the Neolithic to Roman Period in Kea, Greece. More studies at this scale are required to begin to quantify the presence of helminths and by comparing multiple sites, trends over time can be established confidently without the limitation of site specific variation.

1.6 Genetic identification and tracing the dispersal of past pathology

Visual identification of infective agents and the pathology they cause is the dominant tool in the field of archaeology to study health, however, pathogenic organisms are also beginning to be traced by genetics. This is a relatively young discipline due to the low proportion of pathogen DNA that remains in archaeological settings, and the difficulty of distinguishing between environmental microbes and those of epidemiological interest (Tsangaras and Greenwood, 2012). Genetic studies can improve our ability to quantify the presence of infections in historical populations as they can identify infections that cannot be visually detected (see Bedarida et al., 2011).

In addition, genetic analysis can also allow researchers to study routes of infection or the evolution of diseases. There has already been successful studies reconstructing the spread of notable bacteria such as *Yersinia pestis* which causes the plague (Bos et al., 2011; Spyrou et al., 2018) and *Mycobacterium tuberculosis* which causes tuberculosis (Bos et al., 2014; Stucki et al., 2016). The global presence of helminths suggest that there has been a long history of dispersal, and the strong dependency of helminths on their hosts means that their infection routes could also be a useful proxy for understanding the migration of humans.

1.6.1 Past studies on the dispersal of helminths

Genetics are the main tool for assessing the dispersal of organisms but, there have been a handful of cases where dispersal of helminths has been traced without it. Parasites that are restricted to specific regions can be used to infer recent migration of people if they are identified outside of their ecological niche. Le Bailly and Bouchet (2010) traced the presence of *Dicrocoelium* sp. globally and historically, noting that this species did not appear in the Americas until the 17th century, first appearing in a British

colony. Due to the zoonotic nature of this parasite, this suggests that goats and sheep were being transported from Europe to America before adapting to novel intermediate hosts. Another example is the presence of Chinese liver fluke on a personal hygiene stick found along the Silk Road. The presence of this helminth indicated long distance migration from southern or eastern China, which has the right marshy environment for the proliferation of Chinese liver fluke, to the northern relay station where they would not naturally occur (Yeh et al., 2016).

In cases where the migration of human hosts is uncertain, helminths can be used as supplementary evidence for or against dispersal. *T. trichiura* is agreed to have been present in Pre-Columbian America. By utilising palaeoclimatic modelling, sea level change predictions and the biological necessity for eggs to be in soil between 17-35 °C, Araújo et al. (1988) suggested this disproved the "Clovis first and only" hypothesis. They argued that the time it would have taken to cross the Beringian land bridge and the climate of the time would have meant that the colonising individuals could not have been reinfected on route. This conclusion is controversial however, as others suggest that biological features such as hypobiosis or potentially the infections of habitat and bedding would be enough to maintain a population of parasites during the peopling of America (Hawdon and Johnston, 1996). Considering that merely the presence of helminths can be used for inferring dispersal, the potential of genetic approaches to this collection of organisms is vast (Morand, 2012).

1.7 Ancient DNA studies on helminths

1.7.1 Targeted PCR approaches to palaeoparasitology

Ancient DNA analysis is now a well-established field of archaeology and yet the number of studies using this approach to study helminths remains low. The earliest studies using genetics for palaeoparasitology used a targeted approach to confirm microscopically identified ova. Using genetics for identification is useful as not all parasites can be identified through light microscopy. This can either be through a breakdown of the identifiable features or alternatively, the morphology of the egg may only be indicative of the genus and not the species. This is problematic for interpretation when there is a difference in life cycle at the species level, for example *Taenia* spp. Genetic data for the identification of helminths has previously been retrieved from latrines (Flammer et al., 2018; Søe et al., 2015), coprolites with and without microscopic evidence of parasites (Iñiguez et al., 2006, 2003; Leles et al., 2008; Loreille et al., 2001), the intestinal tract of mummies (Oh et al., 2015) and, from sediment samples from the pelvic region of individual burials (Oh et al., 2010).

All of the studies above did not use the genetic data produced to infer population dynamics, except Flammer et al. (2018). In a move beyond species identification, variable targets for *T. trichiura* were developed and used to identify connectivity between two Medieval trading ports, Lübeck and Bristol. Considering that the targeted PCR approach is well established, utilising variable targets for species other than *T. trichiura* is a clear next step for palaeoparasitology. *Ascaris* spp. would be the obvious choice due to their prevalence in past and present populations, the robust nature of their eggs and the fact that they are also proliferated by the faecal-oral route. They would therefore provide a complimentary data set to *T. trichiura* for inferences on the spread of these helminths in relation to their human hosts. There are however, other methods for the identification of endogenous helminth DNA that have the capacity to be informative for palaeoparasitological studies.

1.7.2 Further genetic approaches to palaeoparasitology

There are two further published genetic approaches that have been developed and applied to palaeoparasitology. The first, by Côté et al. (2016), utilised Multiplexing PCR with next generation sequencing to rapidly identify a range of helminths in archaeological samples, even in the absence of microscopic evidence. Whilst this is a high throughput approach, multiplexing requires target regions which are short and conserved enough that they can reliably be used to differentiate between species. This minimises the target regions that can be used and the amount of genetic diversity that can be recovered, therefore it is not the most appropriate approach for tracing dispersal or population dynamics. Another approach published by Søe et al. (2018) utilised floatation of combined communal samples, shotgun sequencing after nested PCR then the construction of mitochondrial consensus sequences to investigate phylogenetic affiliations. Whilst the use of shotgunning should be further explored, some of the methodology used in this study is not suitable for assessing genetic variation in helminths.

The first issue is that the starting material utilised in Søe et al.'s (2018) study is very high as sediment from different communal contexts were combined. High starting sediment weight (75-503 g), combined with the high concentration of eggs found in communal samples, results in a high proportion of eggs per extract and this cannot be replicated when working with individual burials or when comparing thin strata within a communal context. This issue is likely to be conflated during the flotation step. Whilst some researchers utilise flotation in ancient samples, Reinhard et al. (1986) found this approach to be insufficient due to the variability of the preservation of the eggs and the fact that the taphonomic changes could either increase, or decrease, the specific weight

of the eggs. It could be argued that flotation of ancient material would result in only the best-preserved eggs being retained for analysis, therefore improving the chance of success, but with low starting soil weight and a low concentration of eggs any loss would likely lead to a high proportion of false negative results. As DNA can be amplified from a single cell (Lasken, 2013), a theoretical alternative to flotation would be the manual separation of a single egg from the surrounding material. This would be incredibly labour intensive for a wide scale analysis and have low rates of success as not every egg recovered would necessarily be well preserved. This approach is therefore unlikely to be used in practice.

One final issue with the methodology used by Søe et al. (2018), is the construction of full mitochondria from mixed samples. By building consensus mitochondria, the genetic diversity recovered will be minimised as only the most common variants will be retained. Whilst potential SNPs and haplotypes were quantified in the paper, they were not analysed phylogenetically. An incomplete picture of the degree of genetic variation in a population will restrict a researcher's ability to reliably reconstruct population dynamics and dispersal. With these points considered, the extraction methodology and data analysis techniques used by Søe are incompatible for working with single graves or small starting soil samples. It will also minimise the degree of genetic variation that can be recovered from ancient samples which may result in misinterpretation of the population history of these helminths. Despite the drawbacks of the methodology developed by Søe, their use of shotgun sequencing has the potential to be a useful tool for palaeoparasitology. Unfortunately, it appears that no other study has applied this sequencing approach for the study of historical helminths.

1.7.3 Limitations to the study of helminth phylogeography

A factor that may be holding back genetic research in palaeoparasitology is the patchy and incomplete nature of modern reference sequences. Many studies that wish to understand the history of population dynamics and migration of a species combine both modern and ancient samples for a holistic understanding of the species of study (for example: Barnett et al., 2009; Svensson et al., 2007; Teacher et al., 2011). Whilst modern samples can be used for inferring past populations, to be completely representative of a species history all past population events would need to leave descendants in the modern population (Orlando and Cooper, 2014). In practice this means that modern samples are better at providing insight into recent population dynamics whilst ancient samples are best for deep history, and, you need both to assess the continuity of ancient and modern populations.

The major advantage of genetic analysis on modern material is the comparative ease of accessing material, certainty of contextual information and the ability to avoid issues that arise from working with damaged and low quantities of genetic material (described in Willerslev and Cooper, 2005). The problem for helminths is that they are no longer endemic in every country, therefore the reference genomes used for comparison will be biased to certain regions which will affect the capacity for researchers to identify genetic connections over space and time. Museums have the potential to fill the gap (Burrell et al., 2015; Herniou et al., 1998) as they have tissue collections from a range of time periods and locations, and the starting biological material is much greater than what can be gathered from an archaeological context. This additional data would be particularly important for improving the capacity for interpretation of ancient sites in non-endemic regions such as Europe.

This literature review shows that genetic approaches in palaeoparasitology have not yet become routine, which may reflect the lack of modern studies on these taxa. There has however, been enough published research to identify key areas for development. The first is to improve the recovery of variable genetic helminth material from sedimentary samples. Currently it is unclear whether the established amplicon sequencing approach is the most appropriate for this purpose. The second is to develop the database of reference genomes for helminths in non-endemic regions so that interpretation of ancient DNA recovered is not limited by a lack of contextual information. By developing these two areas, genetic analysis helminths may be utilised for the assessment of population dynamics and the spread of diseases, moving beyond species confirmation.

1.8 A case example of the limitation of palaeoparasitology- A UK Literature Review

So far, we have identified two limitations of palaeoparasitology that restrict the ability of researchers to explore the epidemiology of helminth in historical populations. These both stem from the strong focus of research solely on the identification of helminth taxa. This has resulted in a lack of publications attempting to build a prevalence rate for these organisms and, a lack of publications attempting to utilise aDNA for reconstructing population dynamics and proliferation of historical helminths. To demonstrate the limitations discussed above, a literature review of papers previously published on helminths in historic Britain was undertaken. Both archaeological and historical evidence has been used to establish what can be confidently stated about the health impacts of helminths in these populations without addressing the limitations highlighted in this chapter.

1.8.1 Archaeological evidence

The earliest paper identifying helminths in the archaeological record within Britain was by Taylor (1955) who identified *Ascaris spp., Trichuris sp.* and *Dicroelium dendriticum* from a pit in Winchester dating to c. AD 1100. This inspired a number of papers and chapters in the 1960-80's but, following this time most references to helminths in the archaeological record comes from site reports. Many of these are documented by Kenward (2009) in his review of invertebrates in archaeology. 168 site report and papers have been collated and a summary of the published work can be seen in the Supplementary (1.12.1). This collection of published work show that helminths have been positively identified from sites that date from Prehistoric to modern times from a range of regions across Britain. All but ten of the studies in this review assessed helminths from mixed contexts such as latrines, concretions, pit/ditch fills and environmental soils. 21 samples in total came from non-communal contexts including the bog body of the Lindow man (Jones, 1986), soil from the pelvis of Richard the Third (Mitchell et al., 2013), skeletal cysts (Price, 1975), grave fills (Carrott et al., 1996b), coprolites (Anastasiou et al., 2012; Carrott et al., 2005; Dainton et al., 1992; Jones, 2010, 1983) and possible coprolites (Bell, 1990).

When the data is combined regardless of context, it shows that 1,906 samples have been assessed for helminths and 605 of these were positive (31.74%). Some of the contexts assessed were not representative of areas where human waste would be uncovered, such as environmental peat samples, cuts or surface accumulations. Despite this, the proportion of positive samples remains relatively high which suggests a degree of pervasiveness of helminth infections over time. Whilst the Kenward report is a great resource for an overview, it does not separate out samples by time, even when a site spans multiple historical periods, nor does it specify the quantity, or types, of eggs found per sample. This report is also focused on just the North of England with a high number of site reports coming from York (65). Of the additional 26 papers and reports added to this review, only 11 quantified the number of samples assessed and the number which were positive. This current list of literature is not exhaustive so there is likely to be more evidence of the ubiquitous nature of helminths in the UK. With a deeper assessment of the literature specific patterns may become apparent however, from the subsection of papers that were read thoroughly for this thesis, it seems likely that only a handful of the reports will provide details beyond what has been reported here.

As well as highlighting the pervasiveness of helminths in the archaeological record, some inference on different species rates can be made. The most frequently recovered helminth was *T. trichiura* followed by *Ascaris* spp. and if both were found, the

latter was usually at a lower concentration. As *Ascaris* spp. are more prevalent in modern populations, it is notable that *Trichuris* sp. were more common in this review. Taylor (1955) suggested that *T. trichiura.* are better adapted for the damp temperate climate of Britain and that cases with high concentrations of *Ascaris* spp. are indicative of material from pigs rather than humans. Whilst this suggestion is regularly mentioned in the literature, it has not been systematically investigated.

Finally, one study used genetic analysis for the study of helminths in historical Britain. Flammer et al. (2018), identified that the majority of *T. trichiura* ITS1 reads from medieval Finzel's Reach, Bristol, and Viking age York fell within the variation found in modern populations. A handful of reads from Bristol were however, more closely related to a distinct clade of reads that were predominantly recovered from medieval Lübeck. This was suggested to reflect the transference of *T. trichiura* from Lübeck to Bristol during trade as both sites represent major medieval trading ports.

1.8.2 Death records

Medical records also document helminths in historical Britain but these are limited to periods where records were kept and helminths were recognised as infective agents. Fountain (1961) used both the London Bill of Mortality and the Registrar-General's Report (both tables are in the Supplementary 1.12.2-3) to begin to assess the number of deaths associated with 'worm' infections between 1630-1880. Whilst the London Bill of Mortality only covers London and there is some uncertainty of what fell under the classification of "worms", the Registrar General's Report covers a broader geographic area and distinguishes between genera of helminth.

In the Registrar General's Report, there appears to be a drop in deaths associated with worms between 1842-1856 and then the numbers stay consistent, see Figure 1.5. This change could be indicative of a decline in extreme infection loads from improvements to health associated with the "sanitary reform" of the period. The most extreme cases of helminth infection can cause deaths but, most cases would not be accounted for in the death records as the most common helminth infection are more likely to be asymptomatic. Trends in mortality rates from worms are therefore related and influenced by overall prevalence, but will not necessarily follow the same trends.

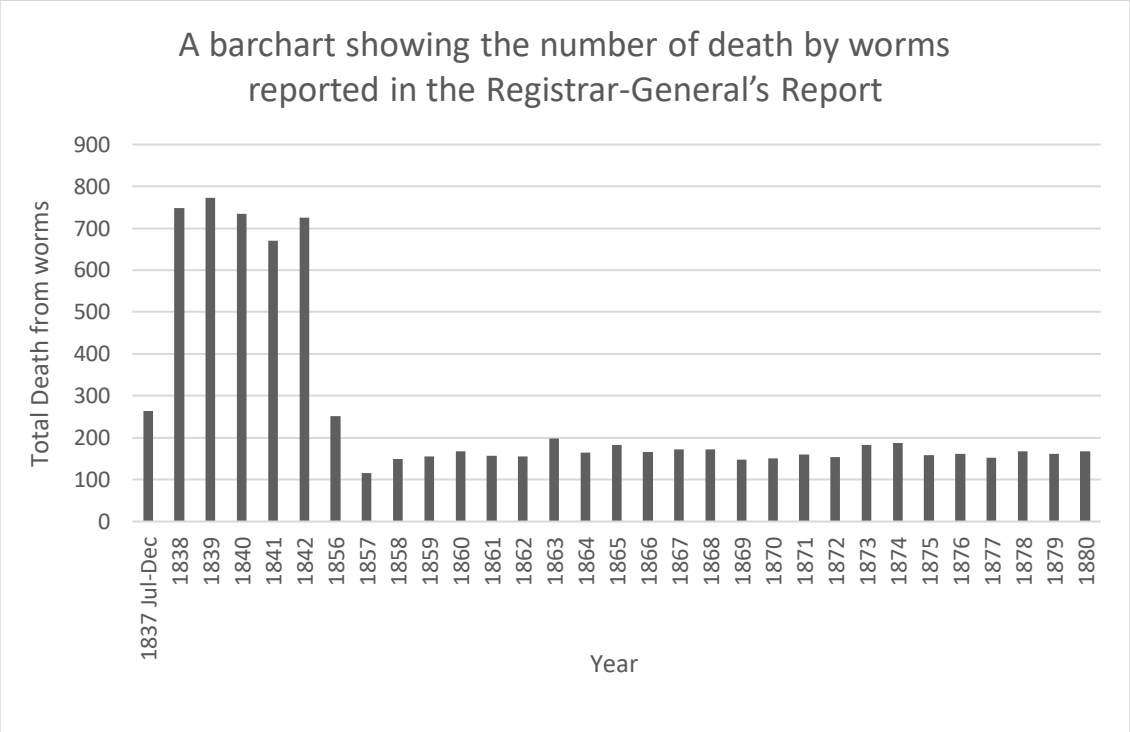


Figure 1.5: A bar chart showing a drop in the number of deaths attributed to worms reported in the Registrar General's Report. Values have been extracted from Fountain (1961). The number of death's reported in 1837 are not for the full year. Between 1843-1846 the records are limited geographically and between 1847-1855 inclusive there is no mention of "worms", therefore there are no values for these periods. A table of this data can be found in the Supplementary 1.12.3.

1.8.3 Hospital records

Hospital records are another source for tracing helminth prevalence. The Historical Hospital Admission Records Project (HHARP) records are freely available online and reflect a handful of children's hospitals dating between 1852-1921 (HHARP, 2018). These records could reflect a higher than average prevalence rate as children are regularly more heavily afflicted with helminths in modern epidemiological studies than adults in terms of prevalence, parasite load and health impacts (Bethony, 2006). These records are predominantly from Great Ormond's Street Hospital but includes some records from Cromwell house, Evelina Hospital and the Royal Hospital for Sick Children, Glasgow.

Of 137,774 total records, 247 (0.18%) were recovered using the search terms: "Helminth", "*Ascaris*", "Worm" and "*Taenia*". The search terms "*Trichuris*", "*Diphyllobothrium*", "*Enterobius*" and "Nematode" did not recover any records. More records were recovered when searching for "debility", "diarrhoea", "enlarged abdomen" and "indigestion" (4695 cases in total, 3.41% of cases) which are all pathologies associated with helminth infection but it is problematic to includes these as these symptoms can also be caused by a number of other infections. Whilst records of worms

were low, the last case of worms dates to 1920 and more specifically the last reference to both *Ascaris* and *Taenia* dates to 1919. Worm cases make up a very small portion of all the records in the HHARP database and there is very little variability over the years with no noticeable trend over time (range from 0-1.81% of between 143-4028 records per year), see Figure 1.6 (all data can be viewed in the Supplementary 1.12.4).

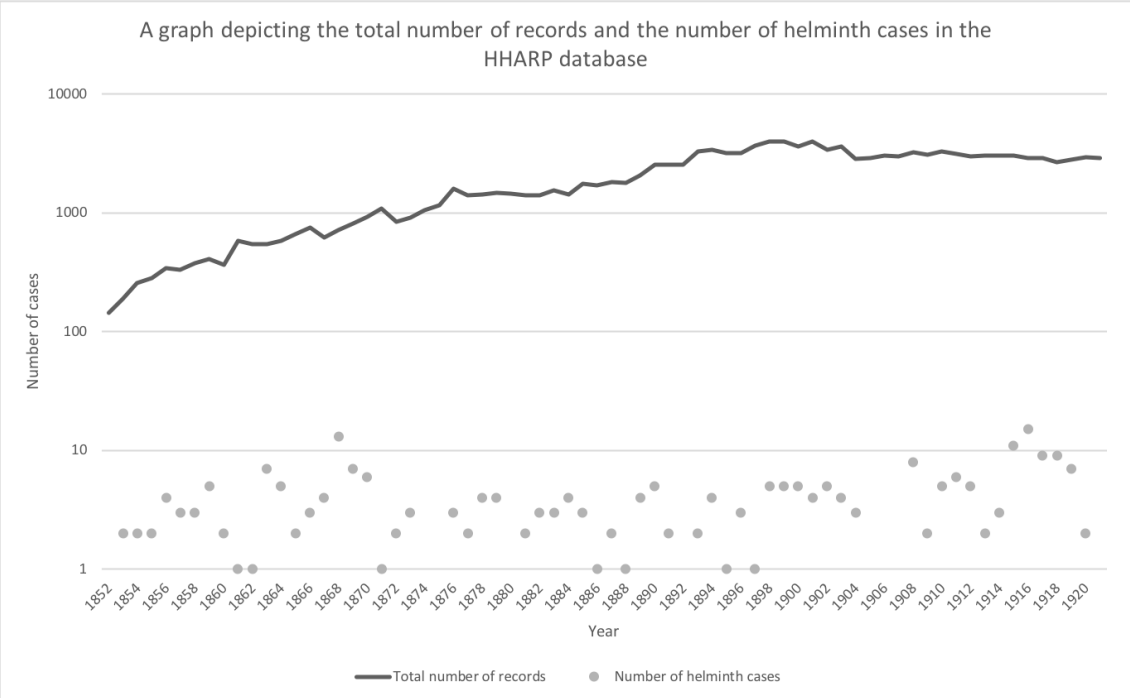


Figure 1.6: A graph depicting the total number of records, and the number of helminth cases recovered from the HHARP database between 1854-1920. The number of cases are on a logarithmic scale so the variation in the number of helminth cases can be visualised. Values have been recovered from HHARP database (2018).

Most of the records recovered were from *Taenia* infections (232, 0.17% of all cases). This is at odds with the archaeological record which predominantly recovered *Trichuris*. This may be due to differential resilience of ova in archaeological contexts or differences in medical detection. The latter falls into two classes: a medical practitioners' ability to detect the worms or, the degree of pathology associated with the different worm infections. *T. trichiura* are small and may go unidentified whereas *Ascaris* spp. worms are large and visible when excreted. They may however, be expelled less regularly than tapeworm segments, which are also clearly visible. Alternatively, *Ascaris* spp. infections have symptoms that are not specific and often mild whereas, *T. solium* can cause cysticercosis which has clear symptoms including seizures or a noticeable cyst under the skin (CDC 2019). Spaces in these hospitals were limited and therefore cases of helminth infections that were mild are unlikely to be represented in this data set.

Finally of note, there is a mention of one individual being treated with an anthelmintic in 1894, but sadly the records do not provide further details of what was used. Buchan (1785) had a number of remedies for expelling worms including: jalap, calomel, aloes, hiera picra, tincture of senna, rhubarb, salad oil, oily clysters, harrowgate water, sea water and Peruvian bark. It is likely that a natural remedy was used in this case as modern antihelmintics were not publicly available until the 20th century. One of the earliest anthelmintic, Piperazine, was first used in the 1950's (Holden-Dye and Walker, 2007). Whilst not all of the treatments cited by Buchan (1785) have been tested, Abdel-Ghaffa et al. (2011) did test the effectiveness of PEG/PC extracts from coconut, onion, garlic, fig, date tree, chicory, ananas, and cistrose and found they had significant killing effects on nematodes, cestodes and trematodes in mice. Natural remedies therefore may have been effective in past populations, but whether the right plants were targeted in this case and administered efficiently is hard to state.

1.8.4 Medical and Public Health papers from the 20th century

Despite the development of modern anthelmintic, there are a number of papers that identify helminth infection in Britain after this time. There appears to be two key groups that these papers identify: children of immigrants and patients in mental health hospitals. There are also a handful of references to other groups including immigrant adults (Constantine et al., 1988), unusual case examples of individuals suffering from extreme pathologies (Haines et al., 1968; Ross, 1942) and infection in rural communities (McNicholl, 1963; Silverston, 1962). The studies on the children of immigrants are based on health assessments prior to the start of school (see Archer et al., 1965; Goel et al., 1977; Thompson et al., 1972). Whilst ethnicity was seen as a risk factor in the earlier studies, the later papers started to focus on how recently the children had gone abroad to endemic regions, and realised that this was the key factor that predicted the likelihood of infection (Fellner and Hall, 1978; Joanes and Srivastava, 1984). Travelling to endemic regions is still a risk factor for helminth infection in Britain today and therefore these childhood studies are not necessarily suggestive of a country that is still endemic.

Many of the studies of the prevalence of helminths in mental health hospitals are opportunistic studies on sudden outbreaks to test the efficiency of novel anthelmintic. The mental health patients participated in some notable behaviours that put these individuals at greater risk. Allen and Green (1989) noted that a number of patients in their study were dirt eaters, smeared faeces and/or were faeces eaters which are all behaviours which would increase the risk of infection. In studies that assessed both patients and staff, staff suffered from a reduced prevalence of helminths (Paine et al., 1960) or were helminth free (Allen and Green, 1989). Whilst these behavioural traits

increase individual risk, it doesn't explain how the eggs ended up in the hospital environment if the country was no longer endemic. Some of the hospitals kept animals within the grounds, whilst others referred to theories that staff from endemic countries were the source of contamination (Lynch et al., 1972). Retrospectively it is hard to state whether these theories hold true.

1.8.5 Conclusions from the UK case study

The current literature on helminths in Britain shows that they have plagued the British population in every period of history right through to the 20th century. Whilst the archaeological record suggests that *T. trichiura,* and to a lesser extent *Ascaris* spp., were the most prevalent in the population, medical records from the 19-20th centuries suggest a higher proportion of *Taenia* spp.. This discrepancy may be a difference in recovery rates between these two resources. Considering the higher prevalence of *Ascaris* spp. and *T. trichiura* than *Taenia* sp. in modern populations, this may suggest that the archaeological records are a more accurate reflection of helminth infections in past populations. One study utilised ancient DNA which suggested that during the Medieval period, a distinct clade of *T. trichiura* spread into Britain through trade links. A much larger study would need to occur however to establish the true distribution of this clade over time and to confirm whether this clade was absent in historical Britain prior to the medieval period.

The death records indicate that people could die of worm infestation however this was a very low proportion of the overall death count. This suggests that extreme pathology associated with infection was infrequent in the past, as it is today. This may partially explain the low number of helminth cases in the HHARP medical records. The death records also show a temporal overlap in the decline of people who died from worms with the sanitary reform movement in the 19th century.

In the following 20th century countries such as America, Japan, Taiwan and the Republic of Korea implemented systematic policies on hygiene infrastructure, health education and wide scale consistent chemotherapy with the goal of controlling helminth infections in these population (Elman et al., 2014; Hara, 1998; Horton, 2003; Kasai et al., 2007; Kobayashi et al., 2006, 2007). Whilst not referred to directly, the absence of published work on government initiatives or research in the UK by the mid-20th century may suggest that helminths were no longer endemic by this point. Helminths were still present in the late 20th century but by this point they were confined to populations with behavioural differences that put them at greater risk (travel or geophagia).

1.8.6 Limitations of the UK case study

Whilst this literature review provides a strongly supported overview of the presence of helminths in historical Britain, modern epidemiological approaches to the data cannot be completed because the numerical values identified here are not suitable as proxies for prevalence rates, nor can values be directly compared between sources. There is a vast number of reports of helminth in archaeological sites dating back to the Neolithic, however 99% (1,885) of the samples studied came from environmental or communal samples. Even the written records are limited. The earliest records here date to 1630, and the death and the hospital records are likely to only reflect the most extreme cases of infection. This means the values identified here will only represent a small proportion of the overall incidence rates. The prevalence rates identified in the school children and mental health patients in the 20th century are also unlikely to be reflective of the broader population due to behavioural differences.

The purpose of studying disease in the past is to use them as natural experiments to understand how social, cultural and environmental changes impact disease. In modern epidemiological studies, variation in prevalence rates of helminths is indicative of differences in infrastructure, education on hygiene practices, agriculture, population density or urbanisation and the degree of migration. Without systematic, reliable quantification of helminths in the past, only limited comparisons can be made between temporally and spatially distinct populations and any inference on external variables that could be at play will be based on speculation. Whilst palaeoparasitology remains focused on identification rather than quantification, inferring the similarities and differences between modern and ancient infection rates of helminths remains difficult despite the large volume of work published in this area.

1.9 Steps to develop palaeoparasitology for tracing health and the spread of disease.

Techniques used for tracing pathogenic organisms in modern studies are not always applicable to historical contexts due to the patchy nature of the archaeological record and the degradation that occurs to the infective bodies over time. This does not mean however that a systematic assessment of the distribution and determinants (causes, risk factors) of helminths should not be the ultimate goal of tracing these pathogens in the past. As discussed above, there are problems with comparing quantitative data on helminths from communal archaeological contexts. This limits the conclusions that can be drawn about the impact of helminth on health in past populations and how this alters with changes to culture and/or environment.

An alternative quantitative approach would be to utilize wide-scale analysis of single burials to produce prevalence rates comparable to modern studies. The degree to which degradation of helminths, along with possible disturbance of the bodies, affects the researcher's capacity to recover count data and significant differences between sites must be explored. This can only be achieved with larger sample sizes than have previously been published. If successful, the first step would be to assess broad trends over space and time before finer scaled inferences, such as the effect of urbanisation on helminth prevalence, can be made.

Another way to improve palaeoparasitological studies for inferences on health is to develop the genetic approaches. The heavy focus on species identification in past publications means that the connectivity of helminths between historical sites is only just beginning to be explored. Understanding the rise and fall of helminth populations, along with the routes of infection, would provide a complimentary data set to support and interpret fluctuations in prevalence rates between sites. Genetics could potentially be used to distinguish whether differences in prevalence rate are actually a reflection of preservation, indicative of an influx of new infected individuals or finally, population growth or decline prompted by a change in the environment.

Variable targets for *T. trichiura* have already been developed (Flammer et al., 2018) however equivalent primers for other species have not currently been published. Alternatively, the shotgunning approach may be a more appropriate tool for the retrieval of genetic helminth data but currently the published protocol by Søe et al. (2018) is limited in the type of contexts that can be studied and the degree of genetic variation that can be recovered. These two approaches need to be explored and compared. In addition, to improve the inferences that can be made from genetic studies of past populations, developments to the genetic approaches will need to coincide with developments to the modern helminth database. This includes resolving the gap left by countries that are no longer endemic. With these genetic techniques developed and combined with systematic wide scale prevalence studies, research on helminths in the archaeological record will begin to be comparable to other infective bodies such as Tuberculosis and Leprosy.

1.10 Overview of thesis

This thesis has four papers exploring how the scope of palaeoparasitology can be improved. Two papers will look at the inferences that can be made when using large single grave datasets. The first is focused on a single time period but from a range of locations to establish the similarities and differences in prevalence rates and risk factors

between Medieval Europe and Modern endemic populations. The second paper focuses instead exclusively on the UK but looks at sites over a range of time periods (Prehistoric through to the Industrial period) to assess whether broad changes to the socio-political landscape impacted the prevalence of helminths.

The next two papers turn to genetic approaches for the development of phylogeography for helminths. The first is a pilot study on the feasibility of using museum collections for reference genomes to fill the gap left in the modern database by non-endemic countries. The final paper will explore genetic techniques that are currently employed in the study of helminths from sedimentary contexts to see whether they can be improved upon, which is the strongest for identifying positive samples and whether either of these tools is appropriate for a wide scale phylogeographic analysis of soil transmitted helminths. This thesis is taking steps to improve our capacity to understand the implication that helminths had on health in historical populations. During the development of these papers, other areas and tools for improvement were identified and are summarised in the concluding chapter.

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1.12 Supplementary

1.12.1 Table- summary of literature review of archaeological evidence of helminths in the UK

Literature review of the published work on helminths in the UK. The first portion has been extracted from Kenward (2009) published tables on the presence of insects in archaeological sites. Additional site reports and papers that were resourced for this thesis are added to the bottom of the table and have been referenced in the bibliography.

Key: B- before, E- early, L- late, M- mid, P- post, nd- not dated, HO-Holocene, LG-Late Glacial, PH- Prehistoric, PL- Palaeolithic, MS- Mesolithic, NE- Neolithic, BA-Bronze Age, IA- Iron Age, RO- Roman, RB- Romano-British, PR- Post Roman, AN-Anglian, AS- Anglo-Scandinavian, SX Anglo-Saxon, MD- Medieval, PM- Post-Medieval, MO- Modern.

Extracted from Kenward 2009 Site reports							
Reference	**Site**	**Location**	**Historical Context**	**Archaeological Context**	**Helminth Type**	**N Tests**	**N Positive**
Carrott and Kenward (EAU 1994/24)	Brook House Farm	Halewood	PH	Ditch fills	*Trichuris* and/or *Ascaris*	2	0
Carrott et al. (EAU 1996/36)	Church Moss	Davenham	PH	Late Devensian and Early Flandrian mire	*Trichuris* and/or *Ascaris*	1	0
Carrott et al. (EAU 1994/31)	St Helens Road, Dringhouses	York	PH, MD	?Pleistocene layer, MD pit and ditch fills, layer	*Trichuris* and/or *Ascaris*	4	1
Carrott et al. (EAU 1994/17)	Brook House Farm + Ochre Brook	Merseyside	PH, RO	ditches and other cuts and some layers	*Trichuris* and/or *Ascaris*	3	3
Carrott et al. (EAU 1994/24)	Brook House Farm + Ochre Brook	Merseyside	PH, RO	ditch fills	*Trichuris* and/or *Ascaris*	2	0
Jones (1986)	Lindow Moss	Wilmslow	PH:IA	Gut of bog body	*Trichuris* and/or *Ascaris*	1	1
Kenward et al. (AML 78/92)	Old Grapes Lane A	Carlisle	?PH, RO-MD	urban fringes; wide range of mostly RO feature types; MD well	*Trichuris* and/or *Ascaris*	76	16
Hall et al. (EAU 2003/14)	Guardian Glass Site	Goole	?MS	peat	*Trichuris* and/or *Ascaris*	1	0
Akeret et al. (PRS 2005/93)	alongside the A63	Melton	NE, EBA, LIA/RB, MD	various features, mainly pit and ditch fills	*Trichuris* and/or *Ascaris*	7	0
Hall et al. (EAU 2004/57)	route of a water pipeline	nr Stamford Bridge	?BA, RB	pit, ditch and grave fills, firing pit, burnt mound	*Trichuris* and/or *Ascaris*	?	1
Carrott et al. (EAU 2004/53)	Aldbrough Gas storage roiect	Aldbrough	IA/RB	Pit and ditch fill	*Trichuris* and/or *Ascaris*	9	4
Akeret et al. (PRS 2005/103)	Faverdale East business Park	Darlington	HO, RB	various deposits, mostly cut fills	*Trichuris* and/or *Ascaris*	10	0

Reference	Site	Location	Historical Context	Archaeological Context	Helminth Type	N Tests	N Positive
Johnstone et al. (EAU 1999/21)	rear of 3 little stonegate	York	1-4th C	turf line, cut fills, surface accumulations	*Trichuris* and/or *Ascaris*	2	0
Mckenna (EAU 1998/30)	Eastgate	Beverley	2-14th C	cut fills and surface deposits; no systematic work on insects	*Trichuris* and/or *Ascaris*	69	34
Carrott et al. (EAU 1998/14)	St Saviourgate	York	early RO-16th C	dumps, pit fills, cut fill	*Trichuris* and/or *Ascaris*	20	14
Carrott et al. (EAU 1998/06)	adjacent to Gibraltar Farm.	Hull	RB	channel, gulley and pit fills; riverbank deposits	*Trichuris* and/or *Ascaris*	2	0
Carrott et al. (EAU 1992/01)	Malmo Road	Hull	RB to MD	RB ditch; MD cuts	*Trichuris* and/or *Ascaris*	1	0
Carrott et al. (EAU 1996/55)	Kingswood	Hull	RB-16th C	ditch, ?ground surface, ?alluvium	*Trichuris* and/or *Ascaris*	4	0
Carrott et al. (EAU 1993/13)	Glebe Farm	Burton-upon-Humber	RB, M-L4th C	pit fills	*Trichuris* and/or *Ascaris*	1	0
Carrott et al. (EAU 1997/17)	Kingswood	Hull	RB, MD	various features	*Trichuris* and/or *Ascaris*	6	0
Buckland (1976a)	Church Street	York	RO	fills of sewer	*Trichuris* and/or *Ascaris*	+	+
Carrott et al. (EAU 1993/10)	All Saints School, Nunnery Lane	York	RO	RO ditch or slot fills	*Trichuris* and/or *Ascaris*	2	0
Carrott et al. (EAU 1994/41)	RAF Catterick	Catterick	RO	pit and ditch fills, layers and other cut and post features	*Trichuris* and/or *Ascaris*	1	0
Carrott et al. (EAU 1995/02)	Dowbridge Close	Kirkham, nr Preston	RO	fort, mainly ditches but a few other features	*Trichuris* and/or *Ascaris*	11	2
Carrott et al. (EAU 2005/53)	3 Driffield Terrence	York	RO	cremation, ditch and pit fills, inhumations	*Trichuris* and/or *Ascaris*	5	0

Reference	Site	Location	Historical Context	Archaeological Context	Helminth Type	N Tests	N Positive
Dainton et al. (EAU 1992/14)	2 St Maurice's Rd	York	RO	ditch fills, coprolite	*Trichuris* and/or *Ascaris*	1	0
Hall et al. (EAU 1994/57)	Cumbris College of Art	Stanwix, Carlisle	RO	putative soil below parade ground	*Trichuris* and/or *Ascaris*	2	0
Hall et al. (EAU 2000/34)	St Thomas street	Scarborough	RO	ditch and cut fills	*Trichuris* and/or *Ascaris*	1	0
Jones (AML 4600)	Ambleside	Ambleside	RO	pit	*Trichuris* and/or *Ascaris*	1	1
Jones and Hutchinson (AML 59/88)	Castle Street	Carlisle	RO	various deposits	*Trichuris* and/or *Ascaris*	16	8
Kenward et al. (AML 76/92)	Old Grapes Lane B	Carlisle	RO	urban fringes; surface deposits and well fills	*Trichuris* and/or *Ascaris*	8	2
Large et al. (EAU 1994/11)	Ribchester	Ribchester	RO	fort, surface deposits and pit and ditch fills	*Trichuris* and/or *Ascaris*	119	21
Schmidl et al. (PRS 2006/47)	Park View School	Chester-le-Street	RO	ditch and cut fills	*Trichuris* and/or *Ascaris*	1	0
Tomlinson (1987)	St Anne's Lane	Nantwich	RO	plank tank fil	*Trichuris* and/or *Ascaris*	1	1
Carrott et al. (EAU 1994/13)	14,18,20 and 22 Back Swinegate	York	RO-16th C	pre-RO, RO military, 11-14 urban occupation and yards	*Trichuris* and/or *Ascaris*	20	5
Hall et al. (EAU 2000/64)	Layerthorpe Bridge	York	RO-19th C	river silts, fills, layers	*Trichuris* and/or *Ascaris*	1	0
Jaques et al. (EAU 2004/46)	25 Bridge Street	Chester	RO-MOD	various deposit types	*Trichuris* and/or *Ascaris*	8	2
Jones and Hutchinson (nd)	Annetwell Street	Carlisle	RO, ?+PR	various depoisits	*Trichuris* and/or *Ascaris*	115	20

Reference	Site	Location	Historical Context	Archaeological Context	Helminth Type	N Tests	N Positive
Hall et al. (EAU 2001/13)	George Hudson Street	York	RO, 11-12, ?14th C	dumps cut fills	*Trichuris* and/or *Ascaris*	2	1
McKenna et al (AML 37/88)	7-9 Aldwark	York	RO, AS, MD	pit fills , layers rampart deposits	*Trichuris* and/or *Ascaris*	73	29
Carrott et al. (EAU 1995/20)	50-52 Monkgate	York	RO, MD	RO and MD ditches, MD pit	*Trichuris* and/or *Ascaris*	4	1
Dobney + Hall (EAU 1992/20)	41 Piccadilly	York	RO, MD	various features	*Trichuris* and/or *Ascaris*	1	0
Jaques et al. (EAU 1999/06)	York Minster Library	York	RO, MD	RO layers, drani, gulley and scoop fills, MD layers	*Trichuris* and/or *Ascaris*	4	0
Kenward et al. (AML 77/92)	Lewthwaites Lane A	Carlisle	RO, MD	urban fringes, RO surface deposits and some cuts; MD pit	*Trichuris* and/or *Ascaris*	18	5
Kenward et al (EAU 1998/32)	Keay's Lane	Carlisle	RO, MD	suburban occupation; wide range of cut and surface feature types	*Trichuris* and/or *Ascaris*	47	10
Kenward et al (EAU 1998/32)	Law's Lane	Carlisle	RO, MD	suburban occupation; various cut and surface feature types	*Trichuris* and/or *Ascaris*	13	4
Carrott et al. (EAU 1995/15)	Carr Naze	Filey	RO, PR	RO signal station, use and abandonment	*Trichuris* and/or *Ascaris*	??7	0
Carrott et al. (EAU 1995/14)	Wellinton Row	York	RO, PR, MD	wide range of deposit types	*Trichuris* and/or *Ascaris*	6	0
Carrott et al. (EAU 1995/53)	22 Piccadilly (=ABC Cinema)	York	RO: 14/15th C	waterfront various deposit types	*Trichuris* and/or *Ascaris*	40	18
Hall and Kenward (1990)	Tanner row & Rougier Street	York	RO:PR:ME:MD	various features See technical reports for assemblage details	*Trichuris* and/or *Ascaris*	293	130
Carrott et al. (EAU 1997/51)	British Gas, Davygate	York	?RO 11-15th C	pit fills, dump	*Trichuris* and/or *Ascaris*	2	2

Reference	Site	Location	Historical Context	Archaeological Context	Helminth Type	N Tests	N Positive
Carrott et al. (EAU 1995/32)	Castle Car Park	York	?RO-Mod	various deposits	*Trichuris* and/or *Ascaris*	4	1
Large et al. (EAU 1998/45)	41 Piccadilly	York	?RO, MD, PM, nd	various deposits , dating vague where invertebrates recovered	*Trichuris* and/or *Ascaris*	2	0
Hall et al. 1992	Coppergate Development	York	PR:SE	helmit pit	*Trichuris* and/or *Ascaris*	1	0
Hall et al. (EAU 2002/16)	Bridge Street	Chester	PRO-20	various deposits	*Trichuris* and/or *Ascaris*	2	2
Johnstone et al. (EAU 1998/42)	Easington	SE of Withernsea	?early SX	pitfill	*Trichuris* and/or *Ascaris*	1	0
Jones (AML 4599)	Coppergate Development	York	AN-AS	pits and surface deposits, some directly associated with structures	*Trichuris* and/or *Ascaris*	2	0
Hall and Kenward (EAU 2000/22)	4-7 Parliament Street	York	AS	layers/dumps, cut fills	*Trichuris* and/or *Ascaris*	4	4
Hall et al. (EAU 1998/30)	All Saints Church, Pavement	York	AS	?external accumulations	*Trichuris* and/or *Ascaris*	4	0
Jones (1983b)	6-8 Pavement	York	AS	Coprolite	*Trichuris* and/or *Ascaris*	1	1
Kenward and Hall (1995)	16-22 Coppergate	York	AS	urban, floors, external surfaces, pits, etc.	*Trichuris* and/or *Ascaris*	+	+
Kenward and Hall (EAU 2000/14)	1-9 Micklegate	York	AS	floors, cut fills, layers	*Trichuris* and/or *Ascaris*	2	2
Kenward and Hall (EAU 2000/20)	188-126 Walmgate	York	AS	floors, alyers, cut fills	*Trichuris* and/or *Ascaris*	21	13
Hall and Kenward (EAU 2000/17)	2 Clifford Street	York	AS (later 9-mid10th C)	surface dumps and layers	*Trichuris* and/or *Ascaris*	1	1

Reference	Site	Location	Historical Context	Archaeological Context	Helminth Type	N Tests	N Positive
Johnstone et al. (EAU 2000/04)	41-49 Walmgate	York	AS, late MD	A/S floors, layers, cut fill; late MD pit fill	*Trichuris* and/or *Ascaris*	8	1
Carrott et al. (EAU 1996/47)	All saints Church	York	AS, MD	Graves, layers	*Trichuris* and/or *Ascaris*	4	0
Carrott et al. (EAU 1997/16)	North Bridge	Doncaster	?AS, 12/13-16/17th C	cut fills and surface deposits	*Trichuris* and/or *Ascaris*	15	0
Hall et al. (EAU 2003/71)	6 St John Street	Beverley	EMD	layer	*Trichuris* and/or *Ascaris*	1	1
KENWARD ET AL. (PRS 2003/50)	28-29 High Ousegate	York	9-11th C	pit fills, surface layers	*Trichuris* and/or *Ascaris*	7	6
Carrott et al. (EAU 1997/24)	47-55 Tanner Row	York	?9th C-modern	layers, cut lining, cut fill; dating vague	*Trichuris* and/or *Ascaris*	1	1
Hall et al. (EAU 2000/80)	rear of 7-15 Spurriergate	York	10-12th C	layers, pit fill	*Trichuris* and/or *Ascaris*	9	6
Carrott et al. (EAU 1992/03)	104-112 Walmgate	York	10-15th C	boreholes, mid 10-mid 11th pits	*Trichuris* and/or *Ascaris*	1	0
Jaques et al. (EAU 2001/26)	41-9 Walmgate	York	10/11-15th C	floors, layers, pit fills	*Trichuris* and/or *Ascaris*	2	1
Carrott et al. (EAU 1994/25)	148 Lawrence Street		11-13th C	urban , cuts and layers associated with structure including leper hospital	*Trichuris* and/or *Ascaris*	6	0
Carrott et al. (EAU 1995/08)	44-5 Parliament Street	York	11-13th C	urban yard surface and pit deposits	*Trichuris* and/or *Ascaris*	7	6
Jaques et al. (EAU 2000/53)	NCP Car park, Skeldergate	York	11-14th C, 16/17th C	pit fills and surface deposits	*Trichuris* and/or *Ascaris*	6	1
Carrott et al. (EAU 1995/21)	Norman Court, Grape Lane	York	11-16th C	urban cut and surface	*Trichuris* and/or *Ascaris*	5	0

Reference	Site	Location	Historical Context	Archaeological Context	Helminth Type	N Tests	N Positive
Carrott et al. (EAU 1998/09)	former Davygate Centre	off Davygate	11/12 -13th C	Samples for insect analysis	*Trichuris* and/or *Ascaris*	3	1
Carrott et al. (EAU 1998/16)	BHS store, Feasgate	York	11/12-12-13th C	floor, pit fills, dumps	*Trichuris* and/or *Ascaris*	6	0
Carrott et al. (EAU 1996/44)	merchat Adventurers Hall	York	11/12-17/18th C	river/pond silts, build-up/levelling	*Trichuris* and/or *Ascaris*	6	4
Carrott et al. (EAU 1998/07)	Near Foredykeand River Hull	Hull	11/13-17th C	ditch fill, fills of garderobe and its outflow, ash spread	*Trichuris* and/or *Ascaris*	2	2
Carrott et al. (EAU 1996/09)	16-22 Coppergate	York	Post Norman Conquest	post Norman conquest occupation deposits	*Trichuris* and/or *Ascaris*	51	32
Carrott et al. (EAU 1995/34)	Old Manor House, Baynard Castle	Cottingham, Hull	12-?14th C	pit/hollow and drain fills	*Trichuris* and/or *Ascaris*	2	0
Carrott et al. (EAU 1995/03)	Keldgate	Beverley	12-13th C	floor pits + others	*Trichuris* and/or *Ascaris*	8	1
Allison et al. (AML 21/90)	Dominican Friary	Beverley	?12-P14th C	landfill;pit;conduit;floors, etc	*Trichuris* and/or *Ascaris*	15	9
Jaques et al. (EAU 2000/59)	The Arcade	Ripon	12-14/15th C	urban cuts and layers	*Trichuris* and/or *Ascaris*	2	1
Carrott et al. (EAU 1993/01)	ChampneyRoad	Beverley	12-15/16th C	floor, silts, pit fills	*Trichuris* and/or *Ascaris*	12	1
Carrott et al. (EAU 1995/35)	1-2 Tower Street	York	12-18th C	Ditch of castle bailey, gully and pit fills	*Trichuris* and/or *Ascaris*	14	6
Akeret et al. (PRS 2005/10)	former Henlys of York filling station	York	12/13-15th C	levelling; demolition, pit fill, alluvium	*Trichuris* and/or *Ascaris*	3	0
Carrott et al. (EAU 1998/10)	Jack Taylor Lane	Beverley	12/13-16/18th C	?natural peat, "deposits", pit fills	*Trichuris* and/or *Ascaris*	5	3

Reference	Site	Location	Historical Context	Archaeological Context	Helminth Type	N Tests	N Positive
Carrott et al. (EAU 2004/43)	NCP car park, Skeldergate	York	12/13, 15/16th C	feature types not evident from report	*Trichuris* and/or *Ascaris*	7	4
Jaques et al. (EAU 2003/63)	Low St Agnesgate	Ripon	12th C	pit fills and surface deposits	*Trichuris* and/or *Ascaris*	1	0
Akeret et al. (PRS 2005/87)	Beverley Health Centre	Beverley	12th C + nd	pit and ditch fills, layer	*Trichuris* and/or *Ascaris*	1	1
Jaques et al. (EAU 2002/06)	Beckside North	Beverley	L12-E13 14-E17th C	various occupation deposits	*Trichuris* and/or *Ascaris*	1	0
Jaques et al. (EAU 2002/12)	St Andrewgate	York	E13-E17th C	floors, pitfills, dumps	*Trichuris* and/or *Ascaris*	7	1
Hall et al. (EAU 1993/26)	63-64 Baxtergate	Whitby	13-15th C	occupation and marine or ditchfill deposits	*Trichuris* and/or *Ascaris*	12	1
Carrott et al. (EAU 1993/05)	North Beckside and Beckview Tilery	Beverley	13-17/18th C	urban pit, garderobe and slot fills, layer, natural deposits	*Trichuris* and/or *Ascaris*	1	1
Carrott et al. (EAU 1994/22)	Cartergate	Grimsby	13-17/18th C	pit and ditch fills and ? Alluvium	*Trichuris* and/or *Ascaris*	6	0
Carrott et al. (EAU 1998/27)	9 Little Stonegate	York	13-17th and nd	various deposits	*Trichuris* and/or *Ascaris*	1	1
Hall et al. (AML 56/93)	The bedern	York	13-17th C + later	mostly pits	*Trichuris* and/or *Ascaris*	147	many
Carrott et al. (EAU 1994/05)	North Bridge	Doncaster	13-18th C	urban buildings and yards	*Trichuris* and/or *Ascaris*	1	1
Akeret et al. (PRS 2005/89)	62-68 Low Petergate	York	13/14, 14-15, ?17. 18-19th C	pit fills, surface deposits	*Trichuris* and/or *Ascaris*	10	7
Carrott et al. (EAU 1996/57)	Hengate	Beverley	13th C-PM	cut fill and surface deposit, no individual dating	*Trichuris* and/or *Ascaris*	2	0

Reference	Site	Location	Historical Context	Archaeological Context	Helminth Type	N Tests	N Positive
McKenna (1987)	High Street and Blackfriargate	Hull	late 13-mid-14th	pit fills, layers, floors	*Trichuris* and/or *Ascaris*	?	present
Carrott et al. (EAU 1994/44)	Yarm	Yarm	MD	ditch fill	*Trichuris* and/or *Ascaris*	5	0
Carrott et al. (EAU 1994/47)	Rosemary Place	York	MD	boreholes penetrating supposed King's fishpool deposits	*Trichuris* and/or *Ascaris*	4	0
Carrott et al. (EAU 1996/15)	44-5 Parliament Street	York	MD	Urban yard surface and pit deposits	*Trichuris* and/or *Ascaris*	40	27
Hall and Kenward (EAU 1990/11)	24-6 The Bolts	Scarborough	MD	occupation deposit	*Trichuris* and/or *Ascaris*	1	0
Hall et al. (EAU 2004/18)	69-73 Morton Lane	Beverley	MD	cut fills occupation layers	*Trichuris* and/or *Ascaris*	2	1
Jones (EAU 1986/34)	HW 79	Chester	MD	pit: concretion	*Trichuris* and/or *Ascaris*	1	1
Jones and Nicholson (AML 229/87)	Tanners Row	Pontefract	MD	pits	*Trichuris* and/or *Ascaris*	10	5
Phillips (1980)	Scale Lane/ Lowgate	Hull	MD	concretion	*Trichuris* and/or *Ascaris*	1	1
Carrott et al. (EAU 1993/07)	Flemingate House	beverley	MD	floor, silt layers, slot and pit fills	*Trichuris* and/or *Ascaris*	3	2
Carrott et al. (EAU 1995/51)	9 St Saviourgate	York	late MD	pit and hollow fills	*Trichuris* and/or *Ascaris*	3	3
Carrott et al. (EAU 1993/08)	Finkle Street and Micklegate	Selby	MD, PM	supposed natural deposits, fish pool, dumps, flood silt, ditch and pit fills	*Trichuris* and/or *Ascaris*	24	7
Jaques et al. (EAU 2004/37)	Sprotbrough Hall Gardens	Sprotborough	MD, PM	pit, ditch, well, culvert, post pipe fills, dumps	*Trichuris* and/or *Ascaris*	4	1
Nicholson et al (AML 41/89)	Coffee Yard	York	MD, PM	floors and associated deposits	*Trichuris* and/or *Ascaris*	32	4

Reference	Site	Location	Historical Context	Archaeological Context	Helminth Type	N Tests	N Positive
Hall et al. (EAU 2000/30)	Foxtons Garage	York	post 13-16th C	deposits	*Trichuris* and/or *Ascaris*	4	0
Alldritt et al. (EAU 1991/01)	17-21 Piccadilly	York	MD-L14th C	boreholes, pits and unidentified, dating mostly poor	*Trichuris* and/or *Ascaris*	1	1
Allison et al. (1996a)	46-54 Fishergate	York	MD-L14th C	pits and surface deposits, some directly associated with structures	*Trichuris* and/or *Ascaris*	56	7
Hall and Kenward (EAU 1992/27)	16-22 Coppergate	York	late 14th C	pit fills	*Trichuris* and/or *Ascaris*	1	1
Carrott et al. (EAU 1994/01)	34A-40 High Street	Hull	14-15th C	urban dumps	*Trichuris* and/or *Ascaris*	4	2
Hall et al. (EAU 2001/15)	St John's Coach Park	York	?14-15th C	build-up'	*Trichuris* and/or *Ascaris*	1	0
Carrott et al. (EAU 1994/49)	37 High Street	Hull	?14th C-LMD	floor, occupation rubbish, cesspit fills	*Trichuris* and/or *Ascaris*	5	2
Carrott et al. (EAU 1995/17)	Magistrates Court site	Hull	14-20th C	pre-occupation, Friary and urban	*Trichuris* and/or *Ascaris*	32	3
Carrott et al. (EAU 1993/02)	St Andrewgate	York	15/16-17/18th C	? Floor and ?build-up	*Trichuris* and/or *Ascaris*	2	0
Carrott et al. (EAU 1993/04)	17-19 St Augustine's Gate	Hedon	15/16-17/18th C	primary ditch fills	*Trichuris* and/or *Ascaris*	2	1
Johnstone et al. (EAU 1999/18)	West Cowick	nr Snaith	15/16-17/18th C	flue pit fills, pre-kiln deposit	*Trichuris* and/or *Ascaris*	8	0
Carrott et al. (EAU 1991/15)	Carmelite Street	York	?-16th C	boreholes, MD surface, ?dumps, ?alluvium 16th dumps	*Trichuris* and/or *Ascaris*	1	0
Akeret et al. (PRS 2004/87)	Hull Central Dry Dock	Kingston upon Hull	16th-20th C	various deposits	*Trichuris* and/or *Ascaris*	1	0

Reference	Site	Location	Historical Context	Archaeological Context	Helminth Type	N Tests	N Positive
Carrott et al. (EAU 1997/21)	Sammy's Point	Hull	17-?19th C	surface deposits and earth closet	*Trichuris* and/or *Ascaris*	1	1
Carrott et al. (EAU 1995/37)	Tower Street	Hull	17th C	ditch and drain fills, ?flood deposits; occupation layers	*Trichuris* and/or *Ascaris*	1	0
Carrott et al. (EAU 2001/06)	Canalside/Witter Place	Chester	18th C	?canal upcast, ?tanning pit fills	*Trichuris* and/or *Ascaris*	3	1
Carrott et al. (EAU 1998/21)	Former Female Prison	York	19th C	"deposit"	*Trichuris* and/or *Ascaris*	4	0
Carrott et al. (EAU 1994/43)	Clifton Mooregate	York	nd	ditch and other cut fills	*Trichuris* and/or *Ascaris*	7	0
Hall and Kenward (EAU 2001/07)	Conesby Quarry Sidings	nr Scunthorpe	nd	borehole samples	*Trichuris* and/or *Ascaris*	4	0
Hall et al. (EAU 1991/23)	20-4 Swinegate	York	nd	no information	*Trichuris* and/or *Ascaris*	1	1
Jones (AML 71/87)	Hunter's Walk	Chester	nd	pitfill	*Trichuris* and/or *Ascaris*	3	3
Jones (AML 237/87)	21-7 Union Terrace	York	nd	pit fills	*Trichuris* and/or *Ascaris*	13	2
Large et al. (EAU 1998/40)	90 Piccadilly	York	nd	deposit	*Trichuris* and/or *Ascaris*	1	0
de Rouffignac 1992	5-7 Foregate Street	Chester	?	occupation deposits	*Trichuris* and/or *Ascaris*	+	+
Hall et al. (EAU 2003/03)	Temple Point	Colton (Nr Leeds)	?	various deposits	*Trichuris* and/or *Ascaris*	4	0
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Dark 2004	Gwent Levels at Goldcliff	South Wales	LMS	Pollen cores	*Trichuris*	ni	ni

Reference	Site	Location	Historical Context	Archaeological Context	Helminth Type	N Tests	N Positive
Bell 1990	Brean Down	Somerset	BA	Amorphous concretions, possible coprolites	*Trichuris* and *Ascaris*	43	13
Carrott 2000	Buiston Aryshire	Scottish Lowland	LBA, AD550-L 7th C	Sediment	*Trichuris*	8	1
Hayes 2006	Buckley's Field	Middlewich	RO	Fill pit		3	0
Knights et al. 1983	Fort. Antonine Wall	Bearsden	RO	Fills. ditches	*Trichuris* and *Ascaris*	ni	ni
Carrott 2004a in Giecco	42-48 Scotch Street	Carlisle	RO-LMD	Pit, cesspit	*Trichuris* and *Ascaris*	4	2
De Rouffignac 1985	Hibernia Wharf	Southwark	RO-MD	Well, Pit	*Trichuris, Ascaris, Taenia* and poss *D. latum*	ni	ni
Flammer et al. 2018		York	AS	Latrine	*Taenia, Trichuris*	1	1
Price 1975		Winchester	SX-MD	Skeletal cysts	*Echinoccocus*	ni	1
Jones 2010	French Quarter	Southampton	SX-PMD	Coprolites/Concretion, Soil	*Trichuris* and *Ascaris*	44	12
Carrott et al. 1996a	The Brooks	Winchester	L10-11th, L13-14th, 14-15th, 15-16th C	Timber lined pitfill, pitfill, ashlar-lined tank converted to well, Pitfill concretion	*Trichuris* and *Ascaris*	ni	ni
Carrott et al. 1994	St John's Hospital	Canterbury	1085-E16th C-E19th C	Latrine	*Trichuris* and *Ascaris*	ni	ni
Beard 1986	Bermondsey Priory	London	L11-E12th C	Latrine	*Ascaris*	ni	ni
Pike & Biddle 1966	Lower Brook Street	Winchester	MD- 11th C	Pit	*Trichuris, Ascaris* and *Dicrocoelium dendriticum*	1	1
Fraser 1981	Perth	Scotland	1150-1300	Dung in Pit	*Trichuris*	ni	ni

Reference	Site	Location	Historical Context	Archaeological Context	Helminth Type	N Tests	N Positive
Taylor 1955	Lower Brook Street	Winchester	12th C	Pit	*Trichuris*, *Ascaris* and *Dicrocoelium dendriticum*	ni	ni
Carrott 2004b in Stevens	Ropetackle	Shoreham-by-Sea	12th-14th C	Pit fills	*Trichuris*, *Ascaris* and Capillaria	4	3
Flammer et al. 2018	Finzel's Reach	Bristol	12-17th C	Waste Deposit ditch	*Trichuris* and/or *Ascaris*	26	25
Gooch 1983		St Albans	13th C	Cess deposit	*Trichuris*	ni	ni
Fraser 1981	Queen street, Aberdeen	Scotland	13-14th C	Sediment	*Trichuris* and *Ascaris*	ni	ni
Pike 1975	Cuckoo lane, High street	Southampton	1300-1350, L 13th C	Cesspit Soil	*Trichuris* and *Ascaris*	ni	ni
Fraser 1981	Elgin	Scotland	14th C	Barrel Sediment	*Trichuris*	ni	ni
Mitchell et al. 2013	Church of the friars minor	Leicester	1485	Sacral sediment samples	*Ascaris*	1	1
Greig 1981		Worcester	15th C	Barrel Latrine	*Trichuris* and *Ascaris*	1	1
Jones 2001	St Patrick's Church	Cowgate, Edinburgh	15th C	Ditch Fill	*Trichuris*	ni	ni
Anastasiou et al. 2012	Spitalfields	London	17-18th C	Coprolites	*Trichuris* and *Ascaris*	5	2
Moffet 1992	Dudley Castle	Dudley, Midlands	PMD	Latrine	Parasite ova present	ni	ni

1.12.2 Table- London Bill of Mortality from Fountain (1961).
** indicates that the data was not separately recorded in the London Bill of Mortality.*

Year	Burials	Died of "Worms"
1630	9237	31
1635	10651	27
1640	11321	*
1645	9608	*
1650	8749	65
1655	11348	*
1660	15118	*
1665	97306	*
1670	20198	*
1675	17244	44
1680	21053	35
1685	23222	17
1690	21461	29
1695	19047	76
1700	19443	53
1705	22097	31
1710	24620	75
1715	22232	44
1720	25454	75
1725	25523	34
1730	26761	15
1735	23538	9
1740	30811	13
1745	21296	13
1750	23727	5
1755	21917	11

1.12.3 Table- Registrar-General's Report from Fountain (1961).
The discrepancy between sex incidence and total incidence is explained by the fact that figures for each sex do not include London while the total does.

Year	Population	Deaths from Worms		
		M	F	Total
1837 Jul-Dec	?	119	145	264
1838	15312256	30	419	749
1839	15515296	342	431	773
1840	15721029	339	374	735
1841	15929492	311	337	671
1842	16123793	329	380	726
1856	19042412	112	139	251
1857	19256516	49	66	115
1858	19471291	61	89	150
1859	19686701	70	85	155
1860	19902713	86	81	167
1861	20119314	56	101	157
1862	20336467	72	84	156
1863	20554137	83	115	198
1864	20772308	77	88	165
1865	20990946	78	105	183
1866	21210020	91	75	166
1867	21429508	81	91	172
1868	21649377	81	91	172
1869	21869607	71	77	148
1870	22090163	71	80	151
1871	22782812	72	88	160
1872	23067835	84	70	154
1873	23356414	87	96	183
1874	23648609	96	92	188
1875	23796072	70	88	158
1876	24145938	71	91	162
1877	24700853	51	101	152
1878	25009867	81	86	167
1879	25273981	72	89	161
1880	25636000	85	83	168

1.12.4 Table- Summary of the reports in the HHARP database between 1852-1921

Year	Total number of records	Taenia cases	Ascaris cases	Worm cases	Tape worm cases	Helminthiasis cases	Total Worm cases	% helminth cases
1852	143						0	0.000
1853	190	2					2	1.053
1854	256	2					2	0.781
1855	281	2					2	0.712
1856	341	4					4	1.173
1857	332	3					3	0.904
1858	380	3					3	0.789
1859	411	5					5	1.217
1860	366	2					2	0.546
1861	578	1					1	0.173
1862	544		1				1	0.184
1863	548	5	2				7	1.277
1864	583	5					5	0.858
1865	658	2					2	0.304
1866	758	3					3	0.396
1867	620	3		1			4	0.645
1868	719	11	1	1			13	1.808
1869	819	5	2				7	0.855
1870	934	5	1				6	0.642
1871	1091	1					1	0.092
1872	846	2					2	0.236
1873	916	3					3	0.328
1874	1047						0	0.000
1875	1156						0	0.000
1876	1592	3					3	0.188
1877	1409		1	1			2	0.142
1878	1432	2		2			4	0.279
1879	1470	3		1			4	0.272
1880	1448						0	0.000
1881	1399	2					2	0.143
1882	1403		3				3	0.214
1883	1552	1		2			3	0.193
1884	1423			3		1	4	0.281
1885	1752	1	1	1			3	0.171
1886	1707	1					1	0.059
1887	1826	2					2	0.110
1888	1790	1					1	0.056
1889	2074	3		1			4	0.193
1890	2562	5					5	0.195
1891	2556	1		1			2	0.078
1892	2551						0	0.000
1893	3313	1	1				2	0.060
1894	3396	4					4	0.118

Year	Total number of records	Taenia cases	Ascaris cases	Worm cases	Tape worm cases	Helminthiasis cases	Total Worm cases	% helminth cases
1895	3216	1					1	0.031
1896	3176	3					3	0.094
1897	3686			1			1	0.027
1898	3981	3		2			5	0.126
1899	3985	5					5	0.125
1900	3617	4	1				5	0.138
1901	4028	3		1			4	0.099
1902	3411	4		1			5	0.147
1903	3634	3		1			4	0.110
1904	2844	2	1				3	0.105
1905	2879						0	0.000
1906	3067						0	0.000
1907	3005						0	0.000
1908	3260	5	2	1			8	0.245
1909	3108	1	1				2	0.064
1910	3300	5					5	0.152
1911	3121	5	1				6	0.192
1912	3000	5					5	0.167
1913	3041	2					2	0.066
1914	3032	3					3	0.099
1915	3037	11					11	0.362
1916	2915	12			3		15	0.515
1917	2914	9					9	0.309
1918	2673	9					9	0.337
1919	2823	6	1				7	0.248
1920	2958				2		2	0.068
1921	2891						0	0.000
Total records	137774	200	20	21	5	1	247	
% of records	100	0.145	0.015	0.015	0.004	0.001	0.179	

Chapter Two: Epidemiological insights from a large-scale investigation of intestinal helminths in medieval Europe

2.1 Abstract

Helminth infections are among the World Health Organisation's top neglected tropical diseases as they have a significant impact on the health of children in developing countries. The widespread presence of parasite eggs in archaeological deposits offers a unique opportunity to study the epidemiology of helminths from past populations prior to the development of modern interventions such as chemotherapy. Herein a large-scale analysis of 589 grave samples from seven medieval European sites is reported, for the development of a baseline rate of infection for this region and time period. Helminth diagnosis was based upon microscopic observation of eggs in samples from the pelvic region of skeletons with species confirmation by PCR of ancient DNA. Two soil transmitted nematodes (*Ascaris* spp. and *Trichuris trichiura*) were identified at all locations and two food derived cestodes, *Diphyllobothrium latum* and *Taenia* spp. at four out of seven sites. The prevalence rates of nematode infection in the medieval populations (1.5 to 25.6% for *T. trichiura*; 9.3-42.9% for *Ascaris* spp.) were comparable to those reported within modern endemically infected populations, whereas the food derived cestodes were found at much lower rates (1.0-9.9%). Sex and age are both risk factors that are explored in modern epidemiological studies and whilst there was some evidence of higher levels of infection in younger individuals, sex was not a defining factor in parasite prevalence in this medieval dataset. The genetic diversity of *T. trichiura* ITS-1 in single graves was variable but much lower than within communal medieval latrine deposits. These results not only show the significance of these ubiquitous infections in medieval Europe, but also provide a baseline for studies on the epidemiology of infection in historical and modern contexts.

2.2 Introduction

Helminths are pathogenic organisms that have been detected throughout history. In modern times, soil transmitted helminths (STH) are a global issue with more than 1.5 billion people affected worldwide, mostly in developing countries. Estimations of the burden of STH infections vary considerably as many studies represent short-term child-focused assessments of the effectiveness of medical intervention within a specific region. Some of the best estimates come from meta-analysis assessing regional prevalence (Chan et al. 1994; de Silva et al. 2003; Pullan et al. 2014). Population-wide prevalence rates also vary according to socio-economic factors, as well as cultural practices and the degree of urbanisation (Parajuli et al. 2014; Faria et al. 2017). Although

rarely life threatening, STH infections are responsible for substantial morbidity in affected populations, being clinically associated with anaemia, malnutrition and developmental effects. This is particularly problematic in children and younger individuals, as they consistently suffer from greater morbidity with a higher prevalence and intensity of infection (Anuar et al. 2014; Jourdan et al. 2017).

Effective anthelmintic drugs were first introduced in the early 1960s and represent the primary intervention for modern control efforts, although this is often supported by provision of improved sanitation, clean water and health education programmes (Ziegelbauer et al. 2012; Strunz et al. 2014; Bieri et al. 2013; Gyorkos et al. 2013). Most areas with endemic helminth infections have experienced some attempt at intervention and the World Health Organisation (WHO) recommends that 75% of school age children in at risk regions should regularly receive chemotherapy. Despite these efforts, helminths remain endemic in many parts of the world.

It has been argued that adults and contaminated environments act as a reservoir for post treatment reintroduction of STH to children, which may limit the effectiveness of chemotherapy if it is not supported by other interventions (Anderson et al. 2015). Perhaps the most notable modern successes were in Japan and the Republic of Korea which were achieved with a combination of anthelminthic campaigns and wide-ranging changes in infrastructure (Hong et al, 2006; Komiya and Kobayashi, 1965; Kobayashi et al., 2006). In modern Europe STH infections are very rare however, reports of infection in archaeological deposits suggest this was not always the case (Bouchet & Le Bailly 2014). Within Europe the decline in STH infections pre-dates the development of modern anthelminthic drugs so studying historic European populations provides a comparator to studies in modern endemic areas.

Helminth eggs are robust and can be identified in a wide range of archaeological samples including the abdominal region of mummified bodies and skeletal remains as well as communal waste pits or latrines using microscopic techniques (Faulkner & Reinhard 2014). Egg morphology is adequate for genus-level diagnosis and this can be supported by molecular approaches to identify species (Flammer et al. 2018; Loreille et al. 2001). A wide range of helminths that infect humans have been detected in archaeological samples including schistosomes, *Fasciola* spp., *Capillaria* spp., *Ascaris* spp., *Trichuris trichiura, Taenia* spp. and *Diphyllobothrium latum* (Bouchet & Le Bailly 2014; Flammer et al. 2018).

Two of the most prevalent STH in modern populations are the nematodes *Ascaris* spp. and *T. trichiura*, which are also two of the most widely reported in archaeological sites (Araújo et al. 2015). The detection of *Taenia* spp. and *D. latum* eggs in archaeological samples has been used to comment on the dietary and culinary practices of past populations (Flammer et al. 2018; Maicher et al. 2017) since transmission to humans involves consumption of uncooked or undercooked fish or red meat. Both of these cestodes have also been described in archaeology, generally at a much lower rate than the nematodes (Nezamabadi et al, 2013; Yeh et al, 2014; Flammer et al, 2018). Most reports of parasites in archaeological deposits deal with a few (or single) burials, or communal deposits therefore the prevalence of infection in historic Europe are unclear.

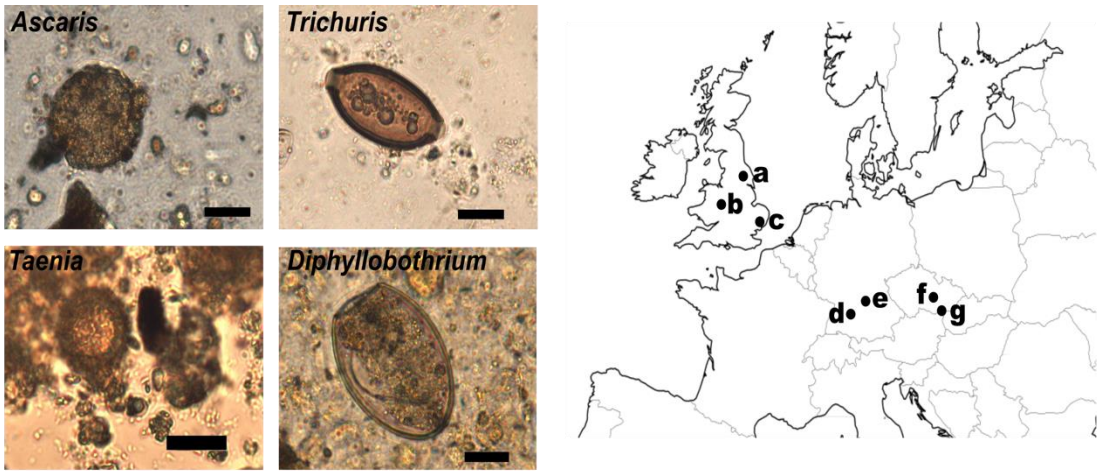
This dataset represents the largest analysis of parasite prevalence in single graves from archaeological contexts and it is the first wide-scale burial analysis from historical Europe. To determine the rates of helminth infection in medieval Europe, 589 single graves were analysed from seven medieval graveyards located in three European countries (UK, Germany and Czech Republic). The sites were; the early Slavic settlement of Břeclav-Pohansko (Czech Republic, 97 samples, Dresler et al. 2016), Ellwangen-Jagst (Germany, 204 samples), Rottenburg Sülchenkirche (Germany, 91 samples, Ade et al. 2018), Ipswich Stoke Quay (UK, 83 samples), York Haymarket (UK, 35 samples), Worchester Cathedral (UK, 63 samples) and Brno Videnska (Czech Republic, 14 samples). The sites date between 680CE and 1700CE.

In addition to estimating historical prevalence rates, metadata associated with these sites allowed us to assess the influence of potential risk factors including age, sex and the estimated population size of the community. To contextualise the results, the data obtained from medieval Europe was compared with those from modern endemic regions (Chan et al. 1994, de Silva et al. 2003, Pullan et al. 2014, see Supplementary 2.8.1 and 2.8.2 for the data used for *Ascaris* spp. and *T. trichiura* respectively). This archaeological dataset represents a period prior to the development of modern control methods, therefore it can be utilised as a pre-modern intervention baseline for future analyses of historical and modern epidemiology to assess the effect of cultural and environmental changes, or human interventions on the prevalence of helminth infections.

2.3 Results

2.3.1 Prevalence of helminth infections in medieval Europe

Helminth eggs were detected by microscopic analysis of samples from the abdominal region of skeletons. An over view of the sites and the helminths detected can be viewed in Figure 2.1. The eggs exhibited well-preserved diagnostic features and four helminths (*T. trichiura*, *Ascaris* spp., *Taenia* spp. and *D. latum*) were identified (representative micrographs in Figure 2.1). Faecal-oral transmitted nematodes *T. trichiura*, *Ascaris* spp. were the most common and detected in all sites. In contrast, the food-associated cestodes *Taenia* spp. and *D. latum* were only detected in samples from four sites (Ellwangen, Pohansko, Rottenburg and Ipswich). In all seven sites, more individuals were infected with *Ascaris* spp. than *T. trichiura* but the prevalence of these nematodes varied between sites. The site with the lowest rates of *Ascaris* spp. and/or *T. trichiura* infection was Worcester (UK) (9.2% and 1.5% respectively) with all other sites having substantially higher rates of infection (Figure 2.1).



	Site	Country	Samples (n)	Dating	*Ascaris* [%]	*Trichuris* [%]	*Taenia* [%]	*Diphyllobothrium* [%]
a	York	UK	35	1100-1600	37.1	8.6	0	0
b	Worcester	UK	65	680-1100	9.2	1.5	0	0
c	Ipswich	UK	83	900-1500	28.9	6.0	7.2	6.0
d	Rottenburg	DE	91	700-1500	22.0	4.4	9.9	2.2
e	Ellwangen	DE	205	700-1700	22.1	7.4	2.5	1.0
f	Pohansko	CZ	97	875-950	35.1	18.6	2.1	2.1
g	Brno	CZ	14	c1400	42.9	28.6	0	0

Figure 2.1: Description of sample origin, summary information and photomicrographs. A map of sampling locations, a table of summary information and photomicrographs representing the four most common parasites with scale bars (20μm).

By combining the data from all sites, *Ascaris* spp. eggs were identified in 25.1% of individuals (range 9.2-42.9%, σ = 10.5%) and *T. trichiura* eggs identified in 8.5% of individuals (range 1.5-28.6%, σ = 8.8%). Within sites where the food–derived cestodes were present, *Taenia* spp. eggs were detected in 3.5% of individuals (range 2.1%-9.9%) with *D. latum* eggs detected in 1.1% of individuals (range 1.0%-6.0%). Co-infection with two or more parasites was detected in 38 of the 589 samples (6.4%). The most frequent co-infection was *T. trichiura* and *Ascaris* spp. (3.9% of samples), followed by *Ascaris*

spp. and *T*aenia spp. (1.2% of samples). Infection with more than two parasites was only seen in three samples (one each of *Ascaris-Trichuris-Taenia, Ascaris-Trichuris-Diphyllobothrium* and *Ascaris-Trichuris-Taenia-Diphyllobothrium*).

2.3.2 The prevalence rates of *Ascaris* spp. and *T. trichiura* in medieval Europe in comparison to modern endemic regions of the world

Although human infection with *Ascaris* spp. and *T. trichiura* are almost completely absent (and mostly travel related) in modern European populations, these infections are endemic in many developing countries. The prevalence rates within medieval European sites were comparable to those reported for regions of the world where soil transmitted helminths are considered endemic, including Africa, Asia and South America (Figure 2.2). The modern prevalence rates were calculated from data presented in three meta-analyses covering the periods 1973-1993 (Chan et al. 1994), 1994-2003 (de Silva et al. 2003) and 2004-2014 (Pullan et al. 2014).

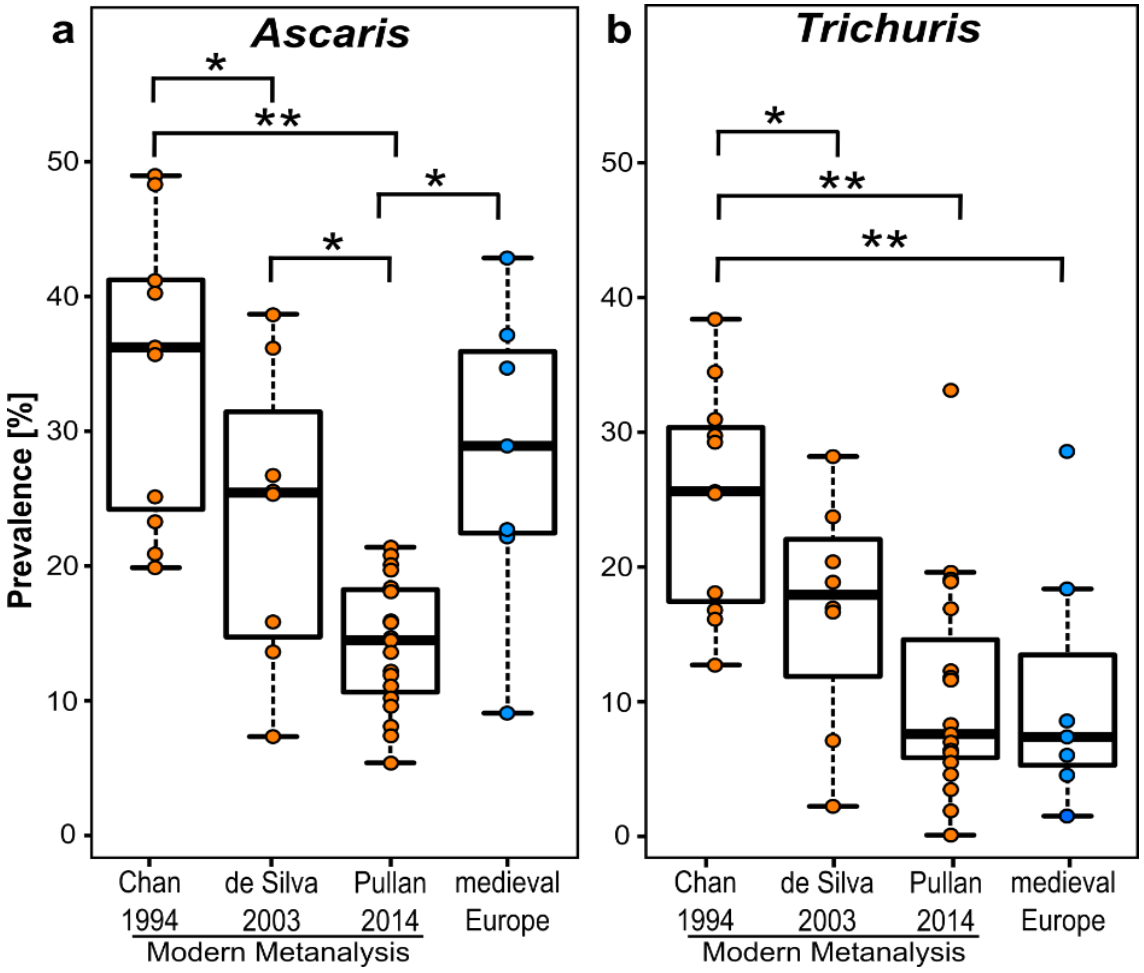
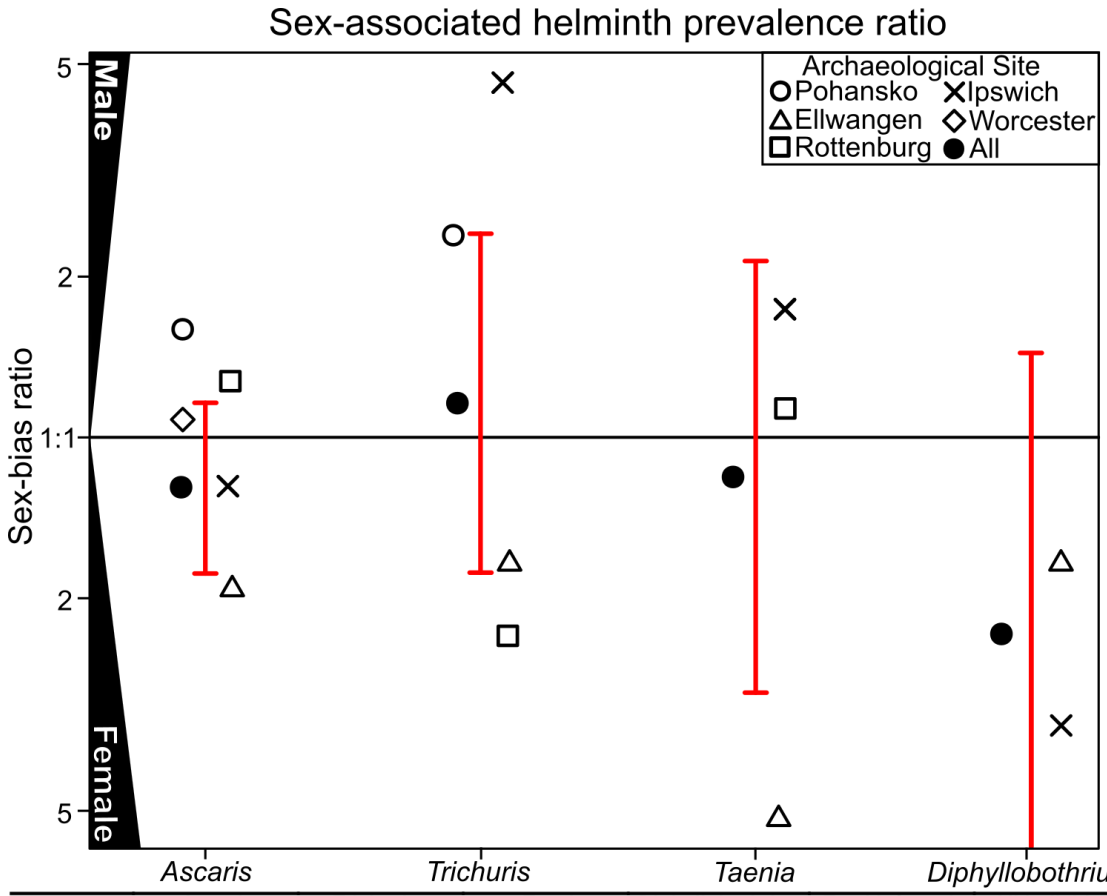


Figure 2.2: Comparison of prevalence rates of Ascaris and Trichuris between modern and medieval populations.
*A boxplot comparing the prevalence rates of Ascaris spp. (a) and T. trichiura (b) of this study's archaeological sites and regional prevalence rates published by Chan 1994, de Silva 2003 and Pullan 2014. Brackets indicates a significant difference using a two-tailed t-test. *= 95% significance level **=99% significance level.*

For both *Ascaris* spp. and *T. trichiura* the mean global prevalence of infection in endemic countries has reduced over the past 30 years with fewer regions reporting prevalence rates greater than 20% in the 2004-2014 reporting period compared with earlier periods. The medieval European prevalence rates for *Ascaris* spp. infection (mean 25.1% of individuals, range 9.2-42.9%, σ = 10.5%) were similar to those reported by Chan et al. (1994) but was significantly higher than those reported by Pullan et al. (2014) (using a two-tailed t-test, 95% significance level). In contrast, the prevalence rates for *T. trichiura* infection in the medieval samples (mean 8.5% of individuals, range 1.5-28.6%, σ = 8.8%) were not significantly different to the rates reported by Pullan et al. (2014) or de Silva et al. (2003) but lower than those reported in Chan et al. (1994) (using a two-tailed t-test, 99% significance level).

2.3.3 Prevalence rates in males and females

Five of the medieval sites provided identification of sex for the majority of adult skeletons (Figure 2.3). The analysis of sex-based prevalence rates was restricted to adults since skeleton-based sex identification is considered unreliable for sub-adults (Meindl et al., 1985). In total, data was available for 378 individuals, with a slight bias towards males (57.1%, n=216) over females (42.9%, n=162). The overall prevalence of parasitic infection was not significantly different between the two sexes (35.8% in females and 29.1% in males; Fisher's Exact two-tailed p-value 0.1825). There was also no evidence for a male: female bias with the prevalence for any of the individual parasites (Fisher's Exact test, female-to-male ratio, 6.8%:7.9% for *T. trichiura*, p=0.843; 25.9%:20.8% for *Ascaris* spp., p=0.267; 4.9%:4.2% for *Taenia* spp., p=0.804 and 4.3%:1.9% for *D. latum,* p=0.217; Figure 2.3). There was a degree of variability between sites but the biggest differences were observed in cases where overall numbers of infected individuals were low within a particular site.



	Ascaris		*Trichuris*		*Taenia*		*Diphyllobothrium*		Total infected		All individuals	
	male	female	male	female	male	female	male	female	male	female	male	female
Ipswich	7	10	4	1	3	2	1	4	12	16	32	37
Pohansko	10	6	5	2	1	0	0	2	11	7	23	22
Rottenburg	9	6	1	2	4	3	2	0	15	13	40	34
Ellwangen	17	19	6	6	1	3	1	1	22	21	95	55
Worcester	2	1	1	0	0	0	0	0	3	1	26	14
Combined	45	42	17	11	9	8	4	7	63	58	216	162

Figure 2.3: Sex-associated helminth prevalence ratio.
A graph depicting the risk ratio of different helminth infections at different sites with 95% confidence intervals for the combined risk ratio. Below is a table to indicate sex based differences for sites where the sample size was too low to depict in this manner.

2.3.4 Prevalence rates by age at death

With modern datasets the prevalence of infection is often reported as differing between pre-school (<5 years), school age (5-18 years) and adults, although prevalence has a weaker association with age than intensity of infection (Bopda et al., 2016; Bundy, 1995; Galvani, 2005). Since the samples contained low egg numbers, count data (to estimate intensity) could be biased by localised post burial disturbance or variable sampling, so the analyses was restricted to prevalence estimates. The metadata associated with five of the sites (Pohansko, Ellwagen, Rottenburg, Ipswich and Worcester) contained detailed estimates for age at death and the skeletal remains from York were subdivided into juvenile (n=6) and adult (n=29). Age-prevalence data was

categorised as infant/young child (≤5 years), child (6-18 years), younger adult (19-40 years) and older adult (40< years).

Combining data from all sites within a mixed effects linear model revealed a significantly lower prevalence of infection with *Ascaris* spp. in the youngest age category (<5 years) compared with the 6-18 (p=0.004) or 40+ (p=0.009) groups and a higher prevalence of infection with *Ascaris* spp. in the 6-18 age group compared with the 19-40 age group (p=0.026) (Figure 2.4). In contrast, there was no evidence for age associated changes in the prevalence of infection with *T. trichiura*. Despite the bias in skeletal age groups in York, a far higher proportion of juveniles (100%) were positive for *Ascaris* spp. infection than adults (24.1%). The prevalence of *T. trichiura* was much lower and there was a smaller difference (juveniles-16.1% and adults-6.9%). No cestode eggs were detected in the 1-5 age group (0%, n=54) compared with 4 positives in the 6-18 group (5.9%, n=68), 15 in the 19-40 age group (6.6%, n=227) and 14 in the over 40-year olds (11.9%, n=118) however the much lower recovery of these ova makes tracing a significant difference difficult. The detailed age structure of the combined sample set and at each site is given in the Supplementary 2.8.3.

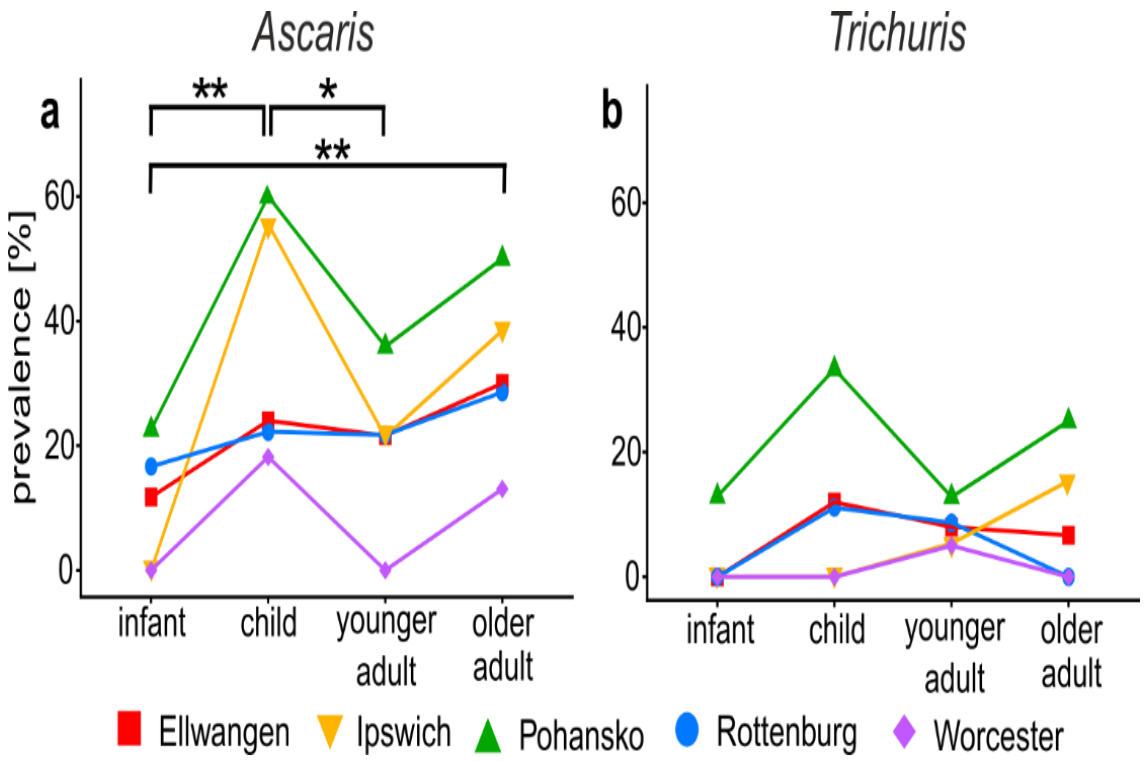


*Figure 2.4: Prevalence of common nematode helminths by site and age group. A graph comparing prevalence of a) Ascaris spp. and b) T. trichiura by age, separated and colour coded by site. Age was classified into four groups: infant (≤5 years), child (6-18 years), younger adult (19-40 years) and older adult (40< years). Brackets indicates a significant difference between age groups when all the data is combined, using a two-tailed t-test. *= 95% significance level **=99% significance level.*

2.3.5 Prevalence rates by population size or historical era

The prevalence rates for infection with the soil transmitted helminths (*Ascaris* spp. and *T. trichiura*) varied between the archaeological sites and this could have been influenced by differences in the size of the local population. The total prevalence rates for *Ascaris* spp. and *T. trichiura* infection were plotted against estimated population sizes for the time and location of the sites (Figure 2.5a, 2.5b). Considering all of the samples, the highest rates for prevalence of infection with *Ascaris* spp. were in York (UK) and Brno (CZ). These sites had the highest population estimates for the sampled time but, also the smallest sample sizes. This pattern was not evident for the prevalence rates of infection with *T. trichiura*. Since there were age-related differences in the prevalence rate of *Ascaris* spp. infections, the data was replotted restricted to just adults (Figure 2.5c, 2.5d). Although Brno remained the site with highest prevalence rate, the adult prevalence rates in York were more similar to those seen with samples from Ipswich, Rottenburg and Ellwangen which had much lower population size estimates, therefore there were no strong correlations between prevalence rates and population size.

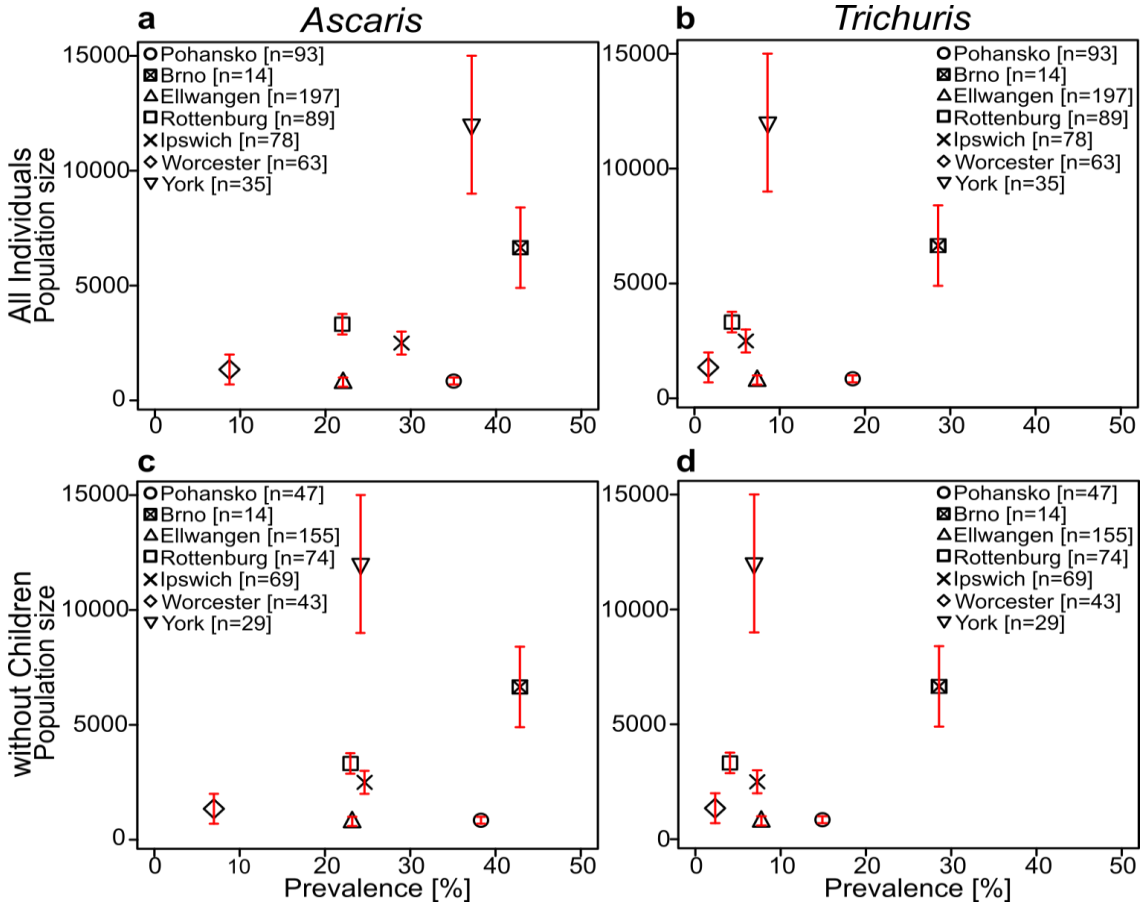


Figure 2.5: Influence of population size on parasite prevalence
A graph plotting the prevalence of a) Ascaris spp. in all samples, b) T. trichiura in all samples c) Ascaris spp. in samples identified as adults and d) T. trichiura in samples identified as adults against each sites estimated population size for the medieval period. Error bars here represent the variation in the estimates of the population size.

Two of the sites had skeletal samples that were individually dated, spanning over the 7-18th centuries for Ellwangen (7-8thc [23 samples], 9-10thc [42 samples], 11-12thc [41 samples], 13-14thc [40 samples], 15-16thc [45 samples], 17-18thc [11 samples]) and 9-15th centuries for Ipswich (9-10thc [14 samples], 11-12thc [33 samples] and 12-15thc [31 samples]). Within Ellwangen (Figure 2.6) and Ipswich (Figure 2.7) the prevalence rates for nematode infections (*Ascaris* spp. and *T. trichiura*) did not significantly vary over time (two-tailed t-test). There were also no significant changes in cestode prevalence over time however, there were periods where they became absent which did not coincide with a drop-in sample size.

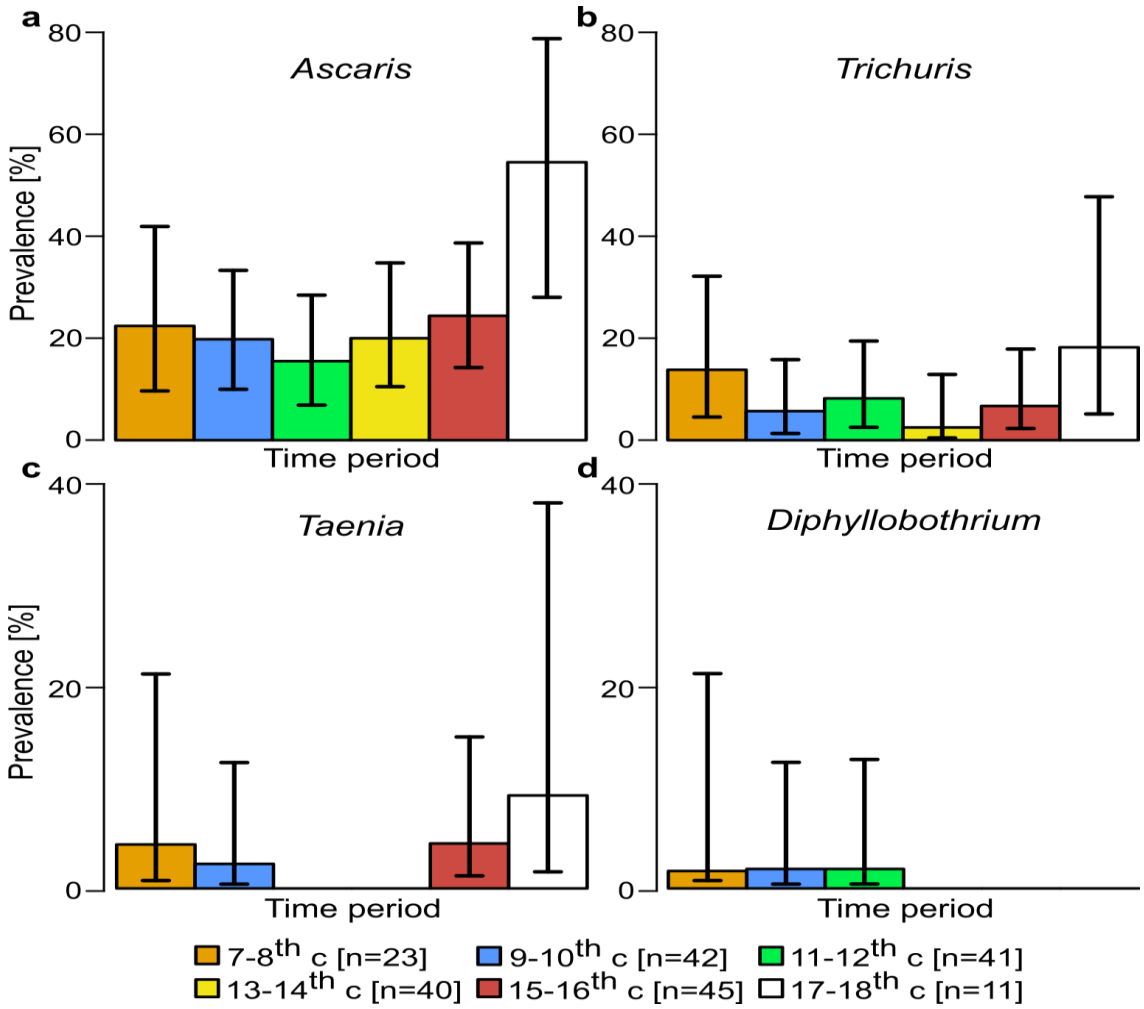


Figure 2.6: Prevalence through time in Ellwangen.
A bar chart showing prevalence over time in Ellwangen. Graves have been grouped by two century intervals. Confidence intervals were calculated using the Wilson Score interval method to a 95% confidence level. Whilst there are some fluctuations over time, they are not significant.

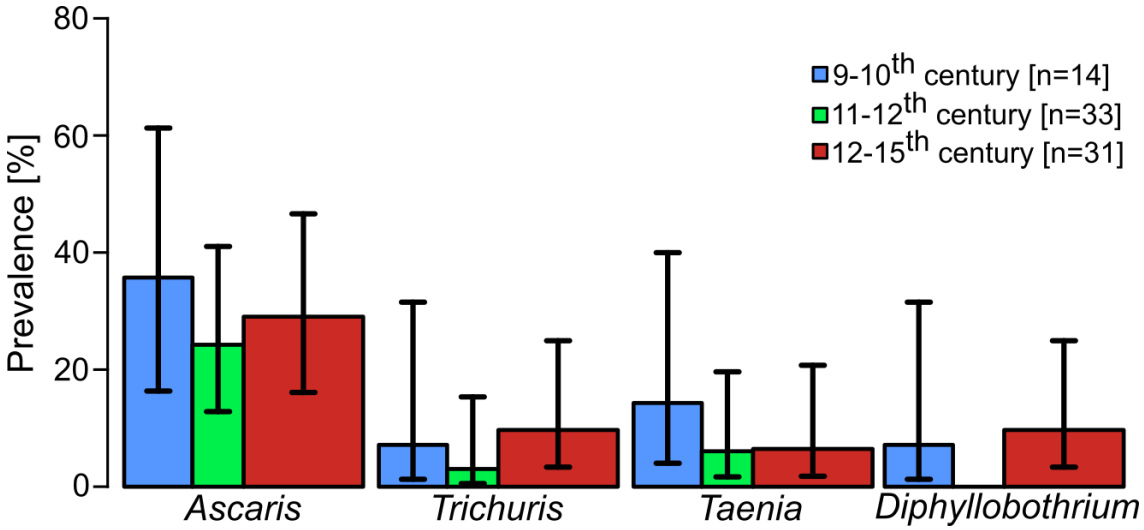


Figure 2.7: Prevalence through time in Ipswich.
A bar chart showing prevalence over time in Ipswich. Graves have been grouped by the time periods provided by the excavating archaeologists, the widths of the bar reflect the length of time. Confidence intervals were calculated using the Wilson Score interval method to a 95% confidence level. Whilst there are some fluctuations over time, they are not significant

2.3.6 aDNA sequencing and genetic diversity

Of the 589 samples, 182 samples containing parasites from Ipswich, Pohansko, Brno, Ellwangen and Rottenburg were selected for aDNA extraction. The samples were subject to PCR and MiSeq sequencing for detection of Trichuris ITS-1 and Ascaris COX-1 fragments using the protocols described in Flammer et al. (2018). Forty-nine single grave derived samples (3 from Ipswich, 18 from Pohansko, 2 from Brno, 17 from Ellwangen and 9 from Rottenburg) yielded at least one positive parasite sequence for *Trichuris trichiura* ITS-1 and/or *Ascaris* COX-1. Six samples generated more than 100 non-singleton reads covering the hypervariable *T. trichiura* ITS-1 fragment which were used to identify the diversity of sequences seen in single grave-derived samples (Figure 2.8a; Po 1-5 and Ip 1).

The level of diversity in single grave derived samples was compared with the diversity of sequences obtained from 13 samples from communal deposits from medieval Bristol (UK) and Lübeck (DE). Each of the six single grave samples were dominated by a single sequence. A single dominant sequence was identified in three of the single grave samples (Po 1, 2 and 5), all four communal samples from medieval Bristol and three of the nine samples from Lübeck (Lu2, 3 and 8). The dominant sequence in Po 4 was identified in Bristol (Br1 and 2) and the dominant sequence in Po3 was present in Br2. The mean and median number of unique *T. trichiura* ITS1 fragment sequences (represented at greater than 1% of total reads) for single graves was lower

(mean 4.0, median 3.0) than with communal samples (mean 8.3, median 9.0). To assess genetic diversity by species richness, the sequencing depth was corrected for by down-sampling the sequence depth to various levels (50, 100, 250, 500, 1000, 2500, 5000, and 10000 reads). Species richness for the single grave samples was not only lower than in communal deposits, but it also did not increase with increasing read depth (Figure 2.8b and 2.8c).

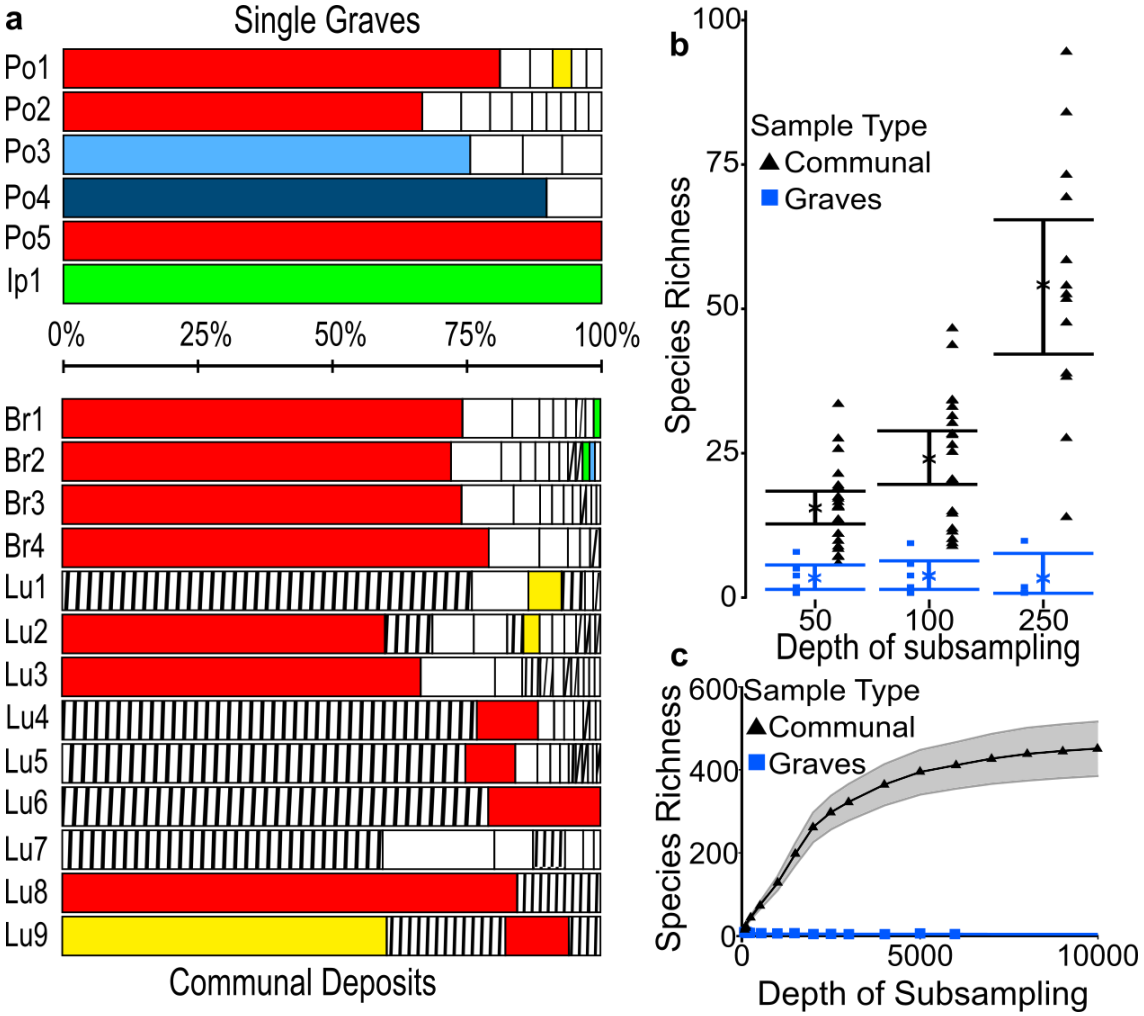


Figure 2.8: Species richness of Trichuris trichiura ITS1 sequences within graveyard population and contemporary communal deposits
a- A visual representation of the haplotypic diversity of reads recovered from different archaeological contexts. The top bars represent data recovered from single burials whilst the bottom bars represent communal contexts. The most common haplotypes of this collection have been coloured to indicate the degree of overlap and variation between the sample types.
b,c- By subsampling we standardised the sampling size and eliminated the sampling bias between datasets. The comparison of species richness yielded a significantly lower species richness in single grave samples. This is expected as the number of contributing parasites will be considerably smaller in a single individual. Graph b represents subsampling depths of 50, 100 and 250 whilst graph c represents subsampling depths of 10000≥.

2.4 Discussion

2.4.1 How do the prevalence rates in medieval Europe differ from modern populations?

The helminth parasites in this study are non-endemic in modern European populations however, a comparison can still be made to other modern populations which are still endemic. The medieval prevalence rate baseline was compared to three meta-analyses of prevalence rates from periods where anthelmintic drugs were available. It is of note, that there was not a global push towards the regular administration of chemotherapy until 2001, endorsed by the World Health Organisation in the WHA 54.19 resolution (Becker et al. 2018). Two of the studies therefore predominantly represent prevalence rates prior to the WHA 54.19 resolution (Chan et al. 1994; de Silva et al. 2003) and one post (Pullan et al. 2014). This study has identified that the medieval prevalence baseline for *Ascaris* spp. overlapped with the prevalence rates of endemic regions prior to the WHA 54.19 resolution, but was significantly different from the meta-analysis of global prevalence rates post this initiative. The inverse pattern was detected for *T. trichiura.*

It is unusual to find disparity in the presence of these two helminth types, as they regularly coinfect due to similarities in their life cycle (both are faecal-orally transmitted helminths), and both have eggs that are robust enough to be regularly found in archaeological contexts (Leles et al., 2010). Whilst both *Ascaris* spp. and *T. trichiura* are able to transmit in a range of environments, they both have unstable transmission with very low, or high, land surface temperatures and high aridity (maximum land surface temperature >38–40°C, mean land surface temperature <10°C, aridity index <0.03 (Pullan & Brooker, 2012)). *Ascaris* spp. differ however, as they are more resistant to extreme temperatures and the larvae can undergo arrested development in the human body for several months, both of which are features that could make *Ascaris* spp. better adapted to the colder and seasonal climate of Europe (Brooker et al. 2006; Pullan & Brooker, 2012).

T. trichiura prevalence rates were lower in medieval Europe than modern endemic regions, and this is likely to reflect the disruption to the life cycle of this taxa due to climatic differences. Modern endemic regions tend to be tropical or sub-tropical, and therefore there is a significant environmental difference between our historical and modern contexts. It is of note that *T. trichiura* have been regularly recovered from communal contexts in Europe (Dark, 2004), but the association between presence in communal contexts and prevalence in single burials has not been investigated, so the significance of these somewhat contradictory finds remains uncertain. The prevalence

of *Ascaris* spp. in medieval Europe on the other hand, does not appear to have been impacted to the same extent by the climatic differences. As the prevalence baseline for *Ascaris* spp. does not appear to be impacted by regional differences, and was high enough to be significantly different from the prevalence rates reported by Pullan et al. (2014), this suggests the WHA 54.19 resolution and modern interventions have had a significant impact on the overall prevalence rates of *Ascaris* spp.

2.4.2 Does consistency in risk factors between medieval and modern data indicate social or biological effects?

The influence of age and sex on prevalence rates was compared, as they are well established risk factors which are regularly explored in epidemiological studies of modern populations. Although some sites showed differences in prevalence between female and male individuals, when the entire sample set was considered, no such correlation could be found. This is consistent with modern data where some populations show a gender bias, but there is no overall trend. There was also increased risk of infection with *Ascaris* spp. in individuals aged between 6-18 in comparison to those aged 1-5 or 19-40. Again, this pattern is found in modern data. To check that external factors were not influencing the results, population size and time period were assessed to establish whether they affected the prevalence of helminths within the medieval dataset. Neither of these factors had a significant impact on the results.

On average, the modern population does not show any sex-associated increase in infection risk with *Ascaris* spp. and *T. trichiura* (Brooker et al, 2004). In cases where sex is a contributing risk factor, this is usually due to a difference in gender roles which may increase the risk of infection, for example, the division of labour. Amongst the indigenous Shuar of Amazonian Ecuador, young males are exposed to higher infection from *T. trichiura* from contact with domestic animals and travel to commercial areas (Gildner et al., 2016), whilst in Vietnam females are the predominant agricultural workers and therefore they have a higher risk of helminth infection, likely from faecal material used as fertiliser (Trang et al., 2007). Gender roles are notoriously difficult to trace in the archaeological record, especially when there are no funerary goods. Based on the sites that provided us with skeletal data, no significant difference in the prevalence rates between males and females in medieval Europe was found, which suggests that there was no universal difference in the gender roles that would alter helminth exposure.

Modern research has consistently found that children are at a higher risk of infection from faecal-orally spread helminths. The increased childhood risk is likely linked to increased exposure to infection (e.g. greater contact to other children, animals and

soil) or absence of acquired immunity (Martins-Melo et al., 2017; Truscott et al., 2014). Depending on which of these hypotheses holds true could affect which strategies are most effective for eliminating helminth morbidity in children. While neither of these theories can be discounted, the consistency of a childhood risk in the medieval population may be indicative of a biological cause rather than a behavioural and environmental one.

Multi-parasitism has been reported to occur in up to 80% of infected individuals in endemic regions (Sayasone et al, 2011). However, infection with more than one species of parasites is not clearly identified as a risk factor as there is neither significant association nor competition between *Ascaris* spp. and *T. trichiura*. Some level of co-infection occurred in this dataset, but never at significant level.

2.4.3 Genetics

The increased availability of aDNA technology has led to improvements in palaeopathology. Genetic information does not only positively identify eggs to the species level, but it also has the potential to reveal helminth infective routes and sources over time. The genetic diversity in single individuals was found to be significantly lower than in communal deposits, however analysis of samples from single burials has the potential to assess the inter-personal differences in parasite infection as the number of different sequences per individual could be used as a proxy to estimate the individual's parasite burden. Finally, the fact that different individuals harboured *T. trichiura* with different ITS-1 sequences does allow us to consider the level of diversity in single skeleton-associated samples which could be used, with further research, as a proxy for estimating the number of human hosts that contributed to the communal samples. Extension of this research will be needed, but this study demonstrates the feasibility and the potential of genetic work on parasites from single individuals.

2.4.4 Can archaeological helminth analysis provide contextual information for modern epidemiology?

In 2001, WHO endorsed the resolution WHA54.19 to tackle helminths using directed chemotherapy interventions to eliminate morbidity in children by 2020 (WHO, 2017). The long-term effects of this resolution on prevalence rates are disputed, especially as adults are not treated (Anderson et al., 2013; Ásbjörnsdóttir et al., 2017; Truscott et al., 2014, Farrell et al, 2018). A literature review of modern case examples found that on average prevalence rates a year after treatment, reached 94% and 82% of pre-treatment levels for *Ascaris* spp. and *T. trichiura* respectively (Jia et al, 2012). This rapid rate of reinfection is due to a combination of adults providing an effective refuge for helminths, in addition to a contaminated environment which usually retains infective

eggs for weeks or months after the anthelmintic treatment (Bopda et al., 2016; Truscott et al., 2014).

The medieval prevalence baseline established in this study suggests that despite these concerns, modern interventions are significantly altering the overall prevalence rates of helminths. It is of note however, that there were some regions, even after the WHA54.19 resolution, which had prevalence rates that were on a par with medieval populations. This suggests that there is a degree of geographical variation in the success of these intervention, which could reflect differences in the implementation of systematic chemotherapy, or differences in the implementation of other complimentary interventions. For example: studies have shown that the effects of deworming are only sustained in conjunction with an improved program of water, sanitation and hygiene (WASH) (Anderson et al., 2013; Freeman et al., 2013; Strunz et al., 2014). This research suggests that if a region has a prevalence rate not significantly different from our medieval prevalence baseline, that the intervention measures in the region are not sufficient. This baseline could therefore be used by modern epidemiologists to identify areas that require the greatest amounts of attention for policy and investment in health.

2.5 Conclusion

Helminth parasites have afflicted mankind throughout human history and remain a significant burden in many parts of the world. Extensive anthelminthic treatment campaigns have been active for decades in many regions, with the aim of reducing the global burden of soil transmitted helminths (STH). A wide range of helminth eggs have been reported in archaeological deposits and these have the capacity to provide comparative case examples to better understand the spread and resilience of these pathogenic organisms. Most previous archaeological studies report parasites in a small number of samples either from single skeletons or communal (e.g. latrine) samples which limit researchers' capacity to quantify and compare the impact of helminths at these sites. As the overall impact of helminth infections on a population is related to the prevalence and intensity of infection rather than their presence or absence in a location, a large-scale analysis of single burials was identified as the most appropriate tool for assessing epidemiological significance of these helminths.

This study determined European medieval prevalence rates by examining 589 skeletal sedimentary samples from seven sites in three countries, representing the largest dataset documenting the prevalence of these infections in any archaeological context. Overall, this dataset has shown that the WHO endorsed resolution WHA54.19 has significantly impacted the prevalence rates of *Ascaris* spp. and that criticisms

suggesting that the intervention has only been limiting the morbidity of these pathogenic organisms do not hold true. The overlap between some modern and medieval prevalence rates at the regional level suggest that there is variation in the reach of chemotherapy and WASH practices, and this medieval baseline could be utilised in the future to identify key areas where the interventions employed are not sufficient.

2.6 Materials and Methods

2.6.1 Sample Material

589 pelvic soil samples were analysed in this study. A comprehensive anthropological analysis was available for the majority of samples. The sites included in this study are the early Slavic settlement of Břeclav-Pohansko (Czech Republic, 97 samples, Dresler et al, 2016), Ellwangen-Jagst (Germany, 204 samples), Rottenburg Sülchenkirche (Germany, 91 samples, Ade et al, 2018), Ipswich Stoke Quay (UK, 83 samples), All Saints in the Marsh, Peasholme; the former Haymarket, York (UK, 35 samples), Worchester Cathedral (UK, 63 samples) and Brno Videnska (Czech Republic, 14 samples). The excavating archaeologists collected the samples on site from the pelvic area of buried bodies.

2.6.2 aDNA extraction

Soil subsamples (~5 g) were re-hydrated in 20 ml of ultra-pure water (Qiagen, Hilden, Germany or SigmaAldrich, Gillingham, UK). The samples were sieved through a series of disposable nylon sieves with decreasing aperture size (1030 µm, 500 µm, 100 µm; Plastok Associates Ltd, Birkenhead, UK) and homogenised with glass beads in a BeadBeater (BSP BioSpec, Bartlesville, USA). aDNA was extracted using Qiagen Blood & Tissue kit (Qiagen, Hilden, Germany).

2.6.3 Microscopic diagnosis

Aliquots of the initial subsample were analysed microscopically using a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL lenses (Nikon UK Ltd., Kingston-Upon-Thames, UK). Photographs were recorded on a QImaging MP5.0 RTV camera (QImaging, Surrey BC, Canada) using the 40x lens (Nikon). Parasite egg counts were extrapolated from replicated counts to the initial dry weight. The subsample used for diagnosis was discarded after microscopy.

2.6.4 Sample handling and preparation workflow

Ancient DNA handling practices have been outlined in a range of publications (Cooper and Poinar 2000, Hofreiter et al. 2001, Pääbo et al. 2004). A specialised workflow was developed to prevent any contamination of samples or aDNA by aDNA extracts, PCR amplicons or unprocessed samples (as described in Flammer et al., 2018). None of the parasites targeted in this study are endemic in the UK or any country

where material was received from. None of the laboratories where samples have been processed have ever handled or stored modern samples containing these parasites, hence modern contamination is unlikely.

The workflow was strictly unidirectional for both material and researchers with no equipment or consumables transferred back between steps or the three physically separate locations where work was undertaken. The laboratory where archaeological samples were initially handled and where aDNA was extracted was an extraction clean laboratory (no PCR, no large quantities of DNA handled). The processing was further confined to a dedicated still air hood (UV2 PCR, Ultra-Violet Products Ltd, Cambridge, UK). The workspace in the still air hood was treated to remove contaminants using a combination of UV irradiation (2 x 30min) and ChemGene HLD4L (Medimark Scientific Ltd, Sevenoaks, UK). Decontamination was performed prior to and after any handling of samples. All PCR was performed in a physically separate, dedicated laboratory space. Where PCR re-amplification was performed these were set up in another dedicated hood (in a second laboratory) decontaminated as described above. All agarose gel analyses and further processing of products occurred in a dedicated space within a third laboratory.

2.6.5 PCR, aDNA sequencing and genetic diversity

Two rounds of PCR were undertaken: the first round used template DNA whilst the second round used 5 μl of the first round PCR solution as the template. The library build consisted of 40 cycles per round, with the Tm of the primers dictating the annealing temperature. Nested primers were used to improve specificity and are based on the primers used in Flammer et al. (2018): ITS1 target for *T. trichiura* and COX1 target for *Ascaris* spp. Gel electrophoresis was used as a preliminary assessment of the success of the amplification. Samples were then selected and a high-fidelity polymerase and further PCR was used to attach the barcodes for MiSeq sequencing (Phusion) before being pooled and cleaned. Parallel sequencing libraries were made from this pooled products and then prepped for sequencing using the NEB- Next Ultra DNA Library Prep Kit for Illumina.

Sequencing was run at the Wellcome Trust Centre for Human Genetics (MiSeq) and the Department of Zoology (MiSeq and Sanger) at the University of Oxford. Sequences were separated according to library barcode using Basespace (Illumina) and then processed using custom python scripts, which identified amplicons with the correct forward and reverse primers, and sorted according to sample barcode.

Sequences were then aligned using MEGA7 (Kumar et al, 2016). MiSeq pair read data was assembled and de-replicated using USEARCH v8 (Edgar and Flyvbjerg 2015).

Samples that yielded at least 100 sequence reads were subsampled to various sequence depths (50, 100, 250, 500, 1000, 2500, 5000 and 10000) using USEARCH v8 (Edgar and Flyvbjerg 2015). To calculate species richness, the R package iNEXT (iNterpolation and EXTrapolation, Chao et al, 2014) was used within R 3.4.2, (R Core Team 2017).

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2.8 Supplementary

2.8.1 Table of the modern prevalence data for *Ascaris* spp. taken from Chan et al. 1994, de Silva et al. 2003 and, Pullan et al. 2014.

Abbreviations includes: SSA (sub-Saharan Africa), LAC (Latin America, Caribbean), MEC (Middle Eastern Crescent), OAI (Other Asia and Islands, without India and China), MENA (Middle East and North Africa), SAS (South Asia), EAP (East Asian and the Pacific Islands).

Publication	Geographical region	Total population [mil]	Infected population [mil]	Prevalence [%]
Chan et al.1994	SSA	512	107	20.90%
Chan et al.1994	LAC	441	182	41.27%
Chan et al.1994	MEC	503	100	19.88%
Chan et al.1994	India	850	198	23.29%
Chan et al.1994	China	1160	568	48.97%
Chan et al.1994	OAI	654	316	48.32%
de Silva et al. 2003	LAC	530	84	15.85%
de Silva et al. 2003	SSA	683	173	25.33%
de Silva et al. 2003	MENA	313	23	7.35%
de Silva et al. 2003	SAS	363	97	26.72%
de Silva et al. 2003	India	1027	140	13.63%
de Silva et al. 2003	EAP	564	204	36.17%
de Silva et al. 2003	China	1295	501	38.69%
Pullan et al. 2014	Asia	3436.7	589	15.8%
Pullan et al. 2014	Central Asia	80.7	6	7.4%
Pullan et al. 2014	East Asia	1424.4	158.4	11.1%
Pullan et al. 2014	South Asia	1621.1	297.8	18.4%
Pullan et al. 2014	Southeast Asia	610.5	126.7	20.8%
Pullan et al. 2014	LAC	586	86	14.7%
Pullan et al. 2014	Caribbean	39.7	3.2	8.1%
Pullan et al. 2014	Andean LA	52.7	10.6	20.1%
Pullan et al. 2014	Central LA	230.3	41.8	18.1%
Pullan et al. 2014	Southern LA	57.9	5.9	10.2%
Pullan et al. 2014	Tropical LA	205.4	24.5	11.9%
Pullan et al. 2014	SSA	866	117.9	13.6%
Pullan et al. 2014	Central SSA	98	21	21.4%
Pullan et al. 2014	East SSA	358.7	34.4	9.6%
Pullan et al. 2014	Southern SSA	70.4	8.6	12.2%
Pullan et al. 2014	West SSA	339	53.9	15.9%
Pullan et al. 2014	MENA	477.4	24.3	5.4%
Pullan et al. 2014	Oceania	9.6	1.9	19.7%

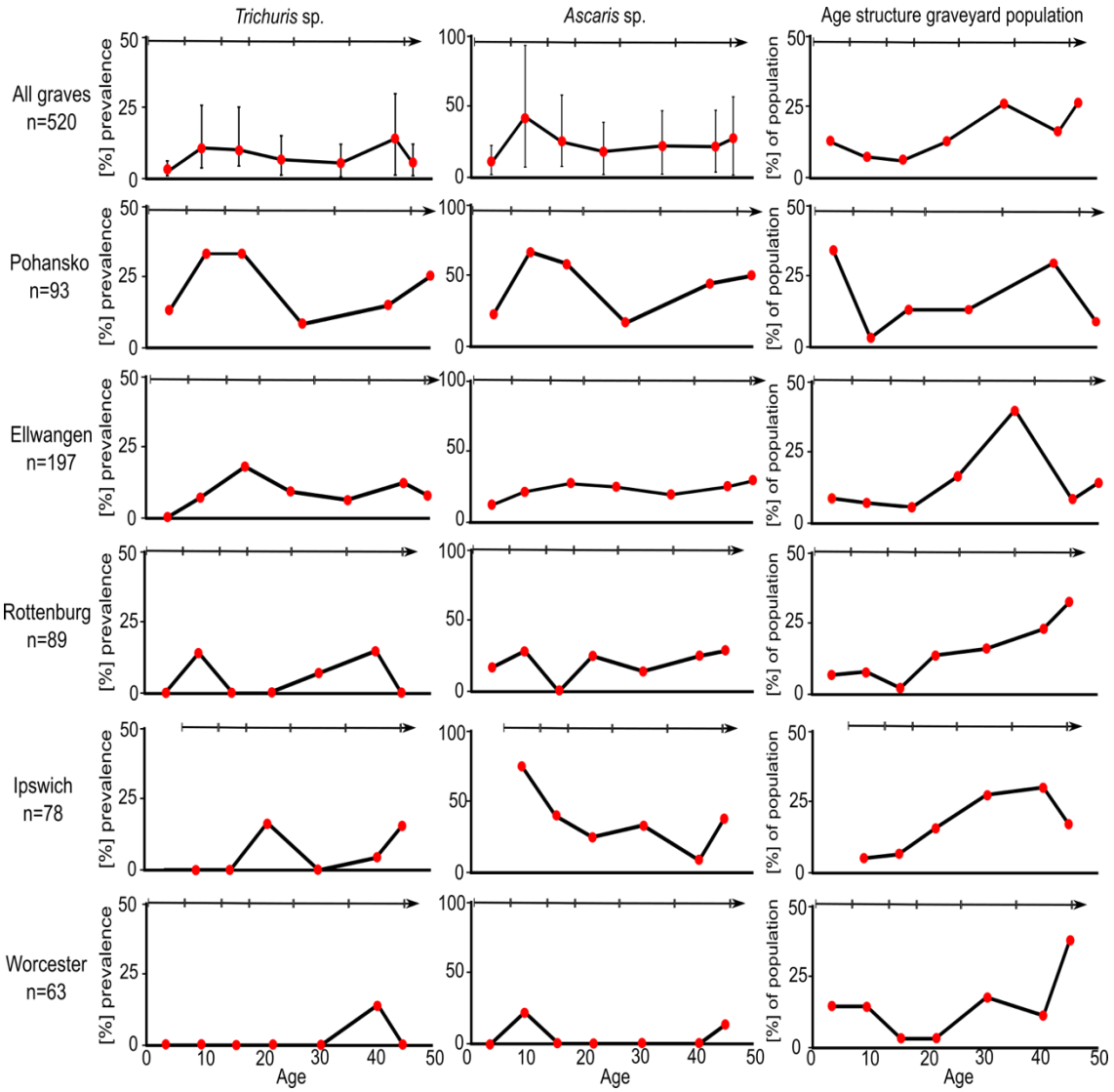
2.8.2 Table of the modern prevalence data for *T. trichiura* taken from Chan et al. 1994, de Silva et al. 2003 and, Pullan et al. 2014.

Abbreviations includes: SSA (sub-Saharan Africa), LAC (Latin America, Caribbean), MEC (Middle Eastern Crescent), OAI (Other Asia and Islands, without India and China), MENA (Middle East and North Africa), SAS (South Asia), EAP (East Asian and the Pacific Islands).

Publication	Geographical region	Total population [mil]	Infected population [mil]	Prevalence [%]
Chan et al.1994	SSA	512	86	16.80%
Chan et al.1994	LAC	441	152	34.47%
Chan et al.1994	MEC	503	64	12.72%
Chan et al.1994	India	850	137	16.12%
Chan et al.1994	China	1160	359	30.95%
Chan et al.1994	OAI	654	251	38.38%
de Silva et al. 2003	LAC	530	100	18.87%
de Silva et al. 2003	SSA	683	162	23.72%
de Silva et al. 2003	MENA	313	7	2.24%
de Silva et al. 2003	SAS	363	74	20.39%
de Silva et al. 2003	India	1027	73	7.11%
de Silva et al. 2003	EAP	564	159	28.19%
de Silva et al. 2003	China	1295	220	16.99%
Pullan et al. 2014	Asia	3436.7	282.3	7.6%
Pullan et al. 2014	Central Asia	80.7	0.1	0.1%
Pullan et al. 2014	East Asia	1424.4	66.2	4.6%
Pullan et al. 2014	South Asia	1621.1	100.7	6.2%
Pullan et al. 2014	Southeast Asia	610.5	115.3	18.9%
Pullan et al. 2014	LAC	586	72.2	12.3%
Pullan et al. 2014	Caribbean	39.7	2.8	7.0%
Pullan et al. 2014	Andean LA	52.7	10.3	19.6%
Pullan et al. 2014	Central LA	230.3	44	19.1%
Pullan et al. 2014	Southern LA	57.9	2.1	3.5%
Pullan et al. 2014	Tropical LA	205.4	13	6.4%
Pullan et al. 2014	SSA	866	100.8	11.6%
Pullan et al. 2014	Central SSA	98	16.5	16.9%
Pullan et al. 2014	East SSA	358.7	42.2	11.8%
Pullan et al. 2014	Southern SSA	70.4	23.3	33.1%
Pullan et al. 2014	West SSA	339	18.8	5.5%
Pullan et al. 2014	MENA	477.4	8.7	1.9%
Pullan et al. 2014	Oceania	9.6	0.6	6.4%

2.8.3 Graph showing the prevalence of common nematode helminths by site and age group.

A graph comparing prevalence by age. Analysis was undertaken by site and with the data combined. The bars on the data combined is the standard deviation to indicate the variation of the prevalence rates between the sites. As skeletal ages were defined as ranges, these are indicated by the arrows whilst the median age was plotted. Whilst some age patterns were apparent within site (Pohansko, Ellwangen and Ipswich), when the data was combined there was no significant difference between age or site.



2.9 Permission from co-authors.
I hereby give permission to Hannah F Ryan to use our joint work as contribution towards her D.Phil. thesis to be submitted for examination at Oxford University.

I confirm that to the best of my knowledge, the author contribution statement below is accurate and Hannah F Ryan's contribution towards the work is greater or equal than that of any other co- author.

Chapter author contribution statement: P.F., H.R., A.S. and G.L. designed the research; P.F. and H.R. collected samples; R.P., B.R., R.N., L.L., C.G., T.W., D.R. and J.W. provided samples and archaeological context; P.F. and H.R. performed microscopic analysis, P.F. performed ancient DNA laboratory work with input from A.S.; P.F. and H.R. produced statistics and graphs from the count data with input from A.S.; P.F. produced statistics and graphs from the genetic data with input from A.S.; A.S. and G.L. secured funding for the project; P.F. and H.R. wrote the initial draft of the manuscript with input from A.S. and G.L.

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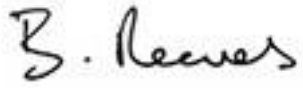
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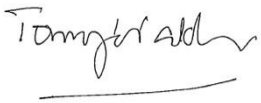
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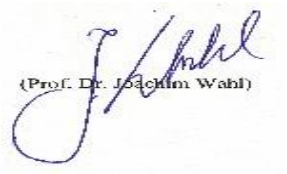
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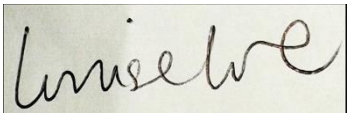
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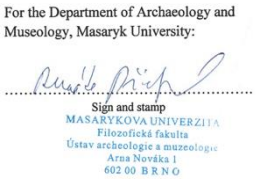
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Chapter Three: Reconstructing Helminth infection rates in Historical Britain - A temporal examination of factors affecting helminth prevalence

3.1 Abstract

Helminths are known to have been an issue in historical Britain as studies on communal and environmental samples provide robust evidence that helminth were persistent from the Prehistoric through to the Industrial period. As the deposition and retention of eggs is variable between these contexts, and there is an unclear connection between the number of eggs deposited and the number of human hosts, there has never been a study of whether there has been a change in the prevalence rate of helminths over time. History provides researchers with the capacity to explore the inter-relationship between changes in culture and their impact on helminths prior to the development of modern WASH practices (Water and Sanitation for Health) and chemotherapy, providing novel epidemiological insight into helminths which can be used to inform modern policy and practices. As communal archaeological contexts are problematic for the quantification of helminths in different historical populations, we cannot assess whether cultural or environmental changes affect helminths unless they result in complete eradication. Here we show that rather than a gradual decline or sudden drop off leading to the eradication of helminths in Britain, there were fluctuations in the prevalence of helminths over time. The environmental and cultural change that had a long-lasting impact on helminths was the "sanitary reform" of the 19th century and the impact of this was felt relatively rapidly in the helminth population, supporting the hypothesis that this was the cause of the eradication of helminths in Europe. Sanitation practices alone however, cannot explain all the fluctuations in prevalence rate detected in this study. Whilst we cannot discount sample bias, this research is a starting point for producing robust epidemiological data for historical periods which can be used to generate well supported evidence of the most significant risk factors for helminth infections in the past.

3.2 Introduction

Studies of ancient pathogens elucidate not only the living conditions of past populations, they also inform our understanding of modern epidemiology. They provide real world instances of distribution and spread of disease (Morand, 2012) which improves our understanding of how pathogens respond to changes in the environment and living conditions (Pullan and Brooker, 2012; Pusch and Czarnetzki, 2005). In practice, this can inform our methods for preventing the spread of diseases (Tognotti, 2013) and show the effectiveness of medical intervention and lifestyle changes on the health of a population. This approach is applicable for enhancing our understanding of

helminths and for informing us of the approaches that can be taken for either the reduction, or eradication of these organisms from the modern population.

Helminth is the generic term for parasitic worms and these organisms are spread through a range of pathways. Much of the medical literature today is focused on soil-transmitted helminths such as *Ascaris* spp. and *Trichuris trichiura,* which are spread via the faecal-oral route of infection but, there is a growing interest in the impact of cestodes, such as *Taenia* spp. and *Diphyllobothrium latum,* which are spread through the consumption of under cooked or under cured meat or fish respectively. Whilst helminth infections can be asymptomatic, in children they can cause inhibition of both mental and physical development, particularly in malnourished populations (Mascarini-Serra, 2011). Due to the impact that helminths have on child development, there is a global initiative to mitigate the morbidity of these pathogens (Becker et al., 2018).

There are a number of countries that, until recently, suffered from high helminth prevalence rates, but the reduction and eventual eradication of these pathogens was achieved through a combination of education, increasing access to sanitation, growing economic wealth and consistent population wide chemotherapy (for example Japan (Kobayashi et al., 2006) and the Republic of Korea (Hong et al., 2006)). The broad-brush approach of these cases masks which of the interventions had the biggest impact on the helminth population, limiting the ability to identify a subset of them to prioritise in endemic regions where a wide scale approach is not feasible. The issue with the broad-brush approach is that it requires high operational and financial capability, as well as political and social capacity and inclination to participate in the changes required (Brooker et al., 2015).

Most European countries have the potential to be used as comparative case examples as they too are no longer considered endemic for the species described above. Unfortunately, these case examples are not as well documented. Currently we know that parasite eggs are found in a large number of archaeological sites from a range of countries and time periods (Bouchet et al., 2003), and it is also widely understood that helminths were eradicated in Europe during the 19-20th centuries, prior to the development of anthelmintics. Anthelmintics are currently the main tool for the limitation of helminths (Becker et al., 2018) but there is debate whether they can provide a long term solution (Anderson et al., 2013; Ásbjörnsdóttir et al., 2017; Truscott et al., 2014).

These European archaeological case examples could provide insight into alternative approaches for the control of helminths, such as Water and Sanitation for

Health (WASH) practices. It is widely understood that helminths were eradicated in Europe due to improved sanitation and infrastructure for the management of waste however, this has not been systematically investigated. WASH interventions have the capacity to limit transmission of helminths, but the results can be variable due to the breadth of factors that fall under the WASH classification. Unfortunately, the scale and the most important types of interventions required to have an impact on the helminth population has not been established (Ásbjörnsdóttir et al., 2017). Despite an abundance of archaeological evidence for the presence of helminths in European archaeological sites (Bouchet et al., 2003), epidemiological methods have not been applied to the data. Any conclusions of how cultural and environmental shifts (such as sanitary improvements, population density, the degree of migration and medical improvements) impacted the helminth population are therefore broad and observational.

Chapter Two has applied epidemiological approaches to archaeological contexts to develop a Medieval prevalence baseline. This was compared to modern epidemiological data to assess the impact of chemotherapy on the prevalence rate of helminths between these two populations. By limiting the comparison to archaeological sites, other variables that impact helminth populations can be more readily explored however, this would require consistent and clear evidence of cultural and environmental differences. An analysis of helminth prevalence over time would have the advantage of well-defined and well supported cultural differences and if analysis was limited to one country, there would be a capacity to assess whether the eradication of helminths was the result of a gradual decline or a sudden drop in prevalence rate. This study selected Britain for the first temporal exploration of the prevalence of helminths in a country that is no longer endemic.

The archaeological record suggests that the British population suffered from helminths from the Prehistoric through to the Industrial period (Kenward, 2009). The most frequently recovered helminths are two of the soil transmitted nematodes (STN) *T. trichiura* and *Ascaris* spp. The majority of studies focus on communal contexts such as latrines, pits and occupational deposits or environmental samples such as peat samples, cuts or surface accumulations. Even in cases where the presence of helminths has been quantified, these studies cannot be used for assessing prevalence rates as the deposition and retention of eggs in communal contexts can differ widely and the association between the number of ova and the number of human hosts cannot be confirmed (Fisher et al., 2007; Jones, 1985), to the point where some researcher argue that quantitative data has very little value (De Rouffignac, 1987). There are a handful of

case examples where the deposition of helminths has a direct association with a single host (for example the Lindow Man (Jones, 1986), King Richard (Mitchell et al., 2013) and the Lloyds Bank coprolite (Jones, 1983)) however there are not enough of these studies to assess change over time. Written records indicate that helminths were still present in Britain in the 19th-20th centuries, however by the mid 20th century helminths were confined to populations that had behavioural differences that put them at greater risk such as travelling to endemic areas (Joanes and Srivastava, 1984) and faeces smearing/eating in mental health hospitals (Allen and Green, 1989).

Despite a wealth of published work on helminths in historical populations (as described in Chapter One), the data either cannot be quantified (from the archaeological records) or represents individuals with the most extreme levels of infection (hospital records, death records). The development of a novel dataset is therefore vital for an epidemiological assessment of helminths in historical Britain. In order to quantify helminths from different time periods, 463 soil samples from the abdomen of skeletal remains in 17 different sites located throughout Britain were analysed, see Figure 3.1. The skeletal remains represent five time periods, Prehistoric, Roman, Anglo-Saxon, Later Medieval and Industrial, and each of these periods is represented by a minimum of two different sites. By assessing the prevalence of helminths in these distinct populations, we can begin to assess whether changes in behaviour and infrastructure associated with WASH practices impacted the helminth population at different time periods, and whether this was the key change to remove these endemic pathogenic organisms.

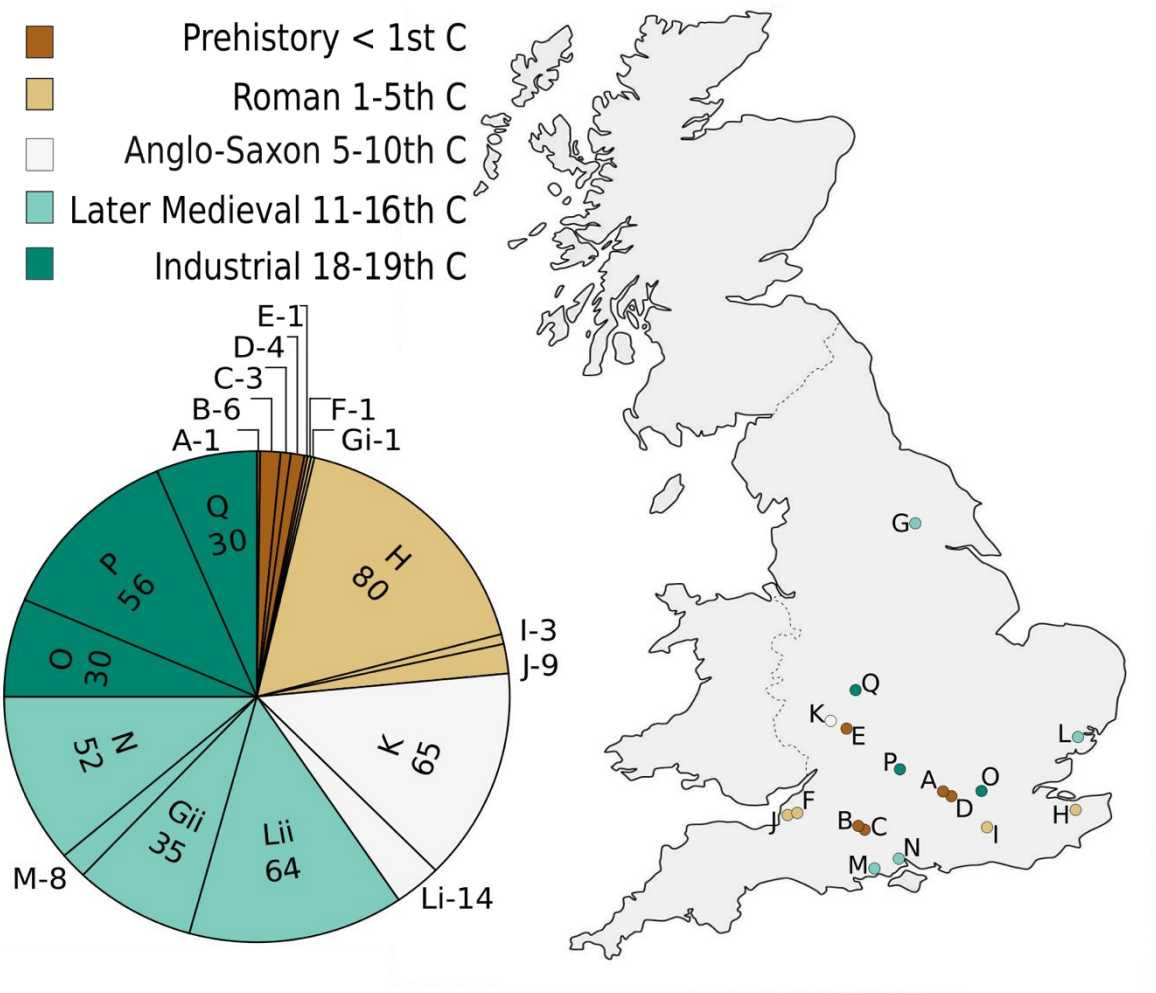


Figure 3.1: A figure depicting the temporal and spatial distribution of the samples used in this study. The pie chart indicates the number and proportion of samples for different time periods and different groups. Each group of samples has been designated a letter: A-Taplow, B-Larkhill, C-Bulford, D-Datchet, E-Vale Crematorium, F-SSSM, Gi-All Saints in the Marsh; Roman, Gii-All Saints in the Marsh; Later Medieval, H-Peugeot Garage, I-North Park Quarry, J-Wentwood Drive, K-Worcester Cathedral, Li-Ipswich Stoke Quay; Anglo-Saxon, Lii-Ipswich Stoke Quay; Later Medieval, M-Christchurch Priory visitors Centre, N-Chapel Riverside, O-St James Burial Ground, P-Radcliffe Infirmary Burial Ground, and Q-Park Street Burial Ground.

3.3 Material & Methods

3.3.1 Material

In this study 17 sites were analysed to represent five different time periods: Prehistoric (Iron Age and earlier), Roman Britain (1st-5th Century), Anglo-Saxon (5th-10th Century), Later Medieval (11th-16th Century) and Industrial (18-19th Century). Sites included Taplow (Prehistoric, 1 sample), Larkhill (Prehistoric, 6 samples), Bulford (Prehistoric, 3 samples), Datchet (Prehistoric, 4 samples), Vale Crematorium (Prehistoric, 1 sample), SSSM (Roman, 1 sample), All Saints in the Marsh (Roman, 1 sample; Later Medieval, 35 samples), Peugeot Garage (Roman, 80 samples), North Park Quarry (Roman, 3 samples), Wentwood Drive (Roman, 9 samples), Worcester

Cathedral (Anglo-Saxon, 65 samples), Ipswich Stoke Quay (Anglo-Saxon, 14 samples; Later Medieval, 64 samples), Christchurch Priory visitors Centre (Later Medieval, 8 samples), Chapel Riverside (Later Medieval, 52 samples), St James Burial Ground (Industrial, 30 samples), Radcliffe Infirmary Burial Ground (Industrial, 56 samples), and Park Street Burial Ground (Industrial, 30 samples), visualised in Figure 3.1. Two of the sites, Ipswich and All Saints in the Marsh, had skeletal remains from multiple time periods so samples from different time periods were grouped separately, resulting in 19 skeletal groups for analysis. Sediment samples were collected from the pelvic region of the skeleton during excavation, except All Saints in the Marsh where samples were taken from sediment adhering to the pelvis of excavated skeletons during the cleaning process. The degree of contextual information that was present for the sites was variable and is summarised in the Supplementary (3.8.1).

3.3.2 Method

To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left-over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. The subsample used for diagnosis was discarded after microscopy. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Confidence intervals for the prevalence rates were calculated using the Wilson Score interval to a 95% confidence level which is recommended in cases of small samples size (Brown et al., 2001) using the Epitools epidemiological calculator (Sergeant, 2019). For the analysis of sex-based differences, the relative risk, standard error and 95% confidence interval was calculated according to the methods described in Altman (1991) using the online MedCalc software (MedCalc Software, 2019). Comparisons of proportions were made either using a two tailed Fisher's exact Test, Welch's T-Test, or the "N-1" Chi-squared test (Campbell, 2007; Richardson, 2011) also using the online MedCalc software. The exception to this was the comparison of the prevalence rates of each group clustered by time period, which was carried out using the Kruskal Wallis Test. To assess whether sample size affected prevalence rates, Pearson's Correlation Coefficient was used. Finally, the archaeological data was also compared to modern data to see how the different time periods compared to a modern

context. The modern data was extracted from three meta-analysis publications which estimate infection prevalence for regions across the globe (Chan et al., 1994; De Silva et al., 2003; Pullan et al., 2014). The data was also explored to isotopic and pathological information that was available. These were small studies which are discussed in Supplementary 3.8.2.

3.4 Results

3.4.1 Overview of the Prevalence Rates in Historical Britain

Of the 19 skeletal groups in this study, 12 had evidence of helminth infections, see Table 3.1. Of the seven groups with no evidence of helminths, all but one of them had a low number of skeletal samples (≤8 skeletal samples). The exception to this was the Industrial Radcliffe Infirmary Burial Ground which consisted of 56 samples. Nematodes (*Ascaris* spp. and *T. trichiura*) were detected in 12 groups whilst the cestodes (*Taenia* spp. and *D. latum*) were detected in six of the groups. If any helminth was detected in a group, the majority of the eggs were *Ascaris* spp. The only group that did not follow this trend was Park Street Burial Ground, from the Industrial period, which had *T. trichiura* at a prevalence rate of 3.33% (n=1) whilst no *Ascaris* spp. were detected. Of the cestodes, *Taenia* spp. were recovered more frequently in five groups whilst *D. latum* was found at a higher rate in one group (St James Burial Ground). The skeletal samples from Later Medieval Ipswich were the only group to have an equal proportion of the two cestodes.

Nine of the 19 skeletal groups had a sample size over 10, and 15 had a sample size over three, as can be seen in Figure 3.1 and Table 3.1. During the assessment of prevalence rate by group, skeletal groups with only 1-2 samples were excluded to mitigate the most extreme effects of small sample size. As can be seen in Figure 3.2, for groups that had a minimum of 3 samples the average prevalence for *Ascaris* spp. was greater (mean=20.52%, median=26.56%, range=0.00-66.67%, σ=20.81%) than for the other three helminth taxa: *T. trichiura* (mean=1.77%, median=0.00%, range=0.00-7.14%, σ=3.00%)), *Taenia* spp. (mean=4.19%, median=0.00%, range=0-33.33%, σ=8.94%), and *D. latum* (mean=1.12%, median=0.00%, range=0-7.14%, σ=2.50%). In all but one case, *T. trichiura*, *Taenia* spp. and *D. latum* were detected in groups which had a sample size greater than 10. The exception was the prehistoric Bulford group where one out of three of the samples contained evidence of *Taenia* spp. To assess whether there was correlation between sample size and prevalence rate of *Ascaris* spp. in the groups, Pearson's correlation coefficient was run. No significant relationship was found (r=0.1225, p=0.6636).

Group Code	Site	Location	No of samples	Date	Period	% Ascaris	% Trichuris	% Taenia	% Diphyllobothrium	% Any Nematode	% Any Cestode	% Sex	Max Age	Min Age	Mid Age	Juvenile: Adults
A	Taplow	Maidenhead	1	Prehistoric	Prehistoric	0.00	0.00	0.00	0.00	0.00	0.00	100			>50	
B	Larkhill	Durrington	6	Prehistoric ?, Late Neolithic-Early Bronze Age	Prehistoric	0.00	0.00	0.00	0.00	0.00	0.00	33.33	35-50	2-12 weeks	13-17	1:0.25
C	Bulford	Salisbury	3	Bronze Age	Prehistoric	33.33	0.00	33.33	0.00	33.33	33.33					
D	Datchet	Slough	4	Neolithic?-Bronze Age	Prehistoric	0.00	0.00	0.00	0.00	0.00	0.00					
E	Vale Crematorium	Worcestershire	1	Prehistoric	Prehistoric	0.00	0.00	0.00	0.00	0.00	0.00					
F	SSSM	Churchill	1	Roman	Roman	100.00	0.00	0.00	0.00	100.00	0.00					
Gi	All Saints in the Marsh	York	1	Civil war period (49-45BC?)	Roman	100.00	0.00	0.00	0.00	100.00	0.00					
H	Peugeot Garage	Canterbury	80	2-3rd C (predominantly)	Roman	35.00	0.00	3.75	1.25	35.00	3.75	53.75	35-45	1-2		1:5.08
I	North Park Quarry	Bletchingley	3	Roman	Roman	0.00	0.00	0.00	0.00	0.00	0.00					
J	Wentwood Drive	Bleadon	9	Roman	Roman	66.67	0.00	0.00	0.00	66.67	0.00					
K	Worcester Cathedral	Worcester	65	c. 680 - c. 1100	Anglo Saxon	9.23	1.54	0.00	0.00	10.77	0.00	65.6	45+	0	-35	1:1.95
Li	Stoke Quay	Ipswich	14	9-10th C	Anglo Saxon	35.71	7.14	14.29	7.14	42.86	21.43	78.57	45+	6-12	36-45	1:2.5
Lii	Stoke Quay	Ipswich	64	11-15th C	Later Medieval	26.56	6.25	6.25	6.25	29.69	12.50	90.625	60+	6-12	26-35	1:11.6
Gii	All Saints in the Marsh	York	35	11(?)-16th C	Later Medieval	37.14	8.57	0.00	0.00	42.86	0.00					1:4.86
M	Christchurch Priory visitors Centre	Dorset	8	11-16th C	Later Medieval	0.00	0.00	0.00	0.00	0.00	0.00	100	60+	35-45	50-60	
N	Chapel Riverside	Southampton	52	13-16th C	Later Medieval	38.46	0.00	1.92	0.00	38.46	1.92	34.62	60+	12-18 months	30-37.5	1:1.6
O	St James Burial Ground	London	30	1788- Mid-19th C	Industrial	26.67	0.00	3.33	6.67	26.67	10.00					
P	Radcliffe Infirmary Burial Ground	Oxford	56	1770-1855	Industrial	0.00	0.00	0.00	0.00	0.00	0.00					
Q	Park Street Burial Ground	Birmingham	30	19th century (1810-1873)	Industrial	0.00	3.33	0.00	0.00	3.33	0.00	70	36-45	1-6 months		1:2.75

Table 3.1: A table depicting the age and location of the different groups on samples along with osteological data and the prevalence of each helminth taxa. Median age is only given if all individuals were designated a numerical age rather than a mixture of descriptive and numerical classifications.

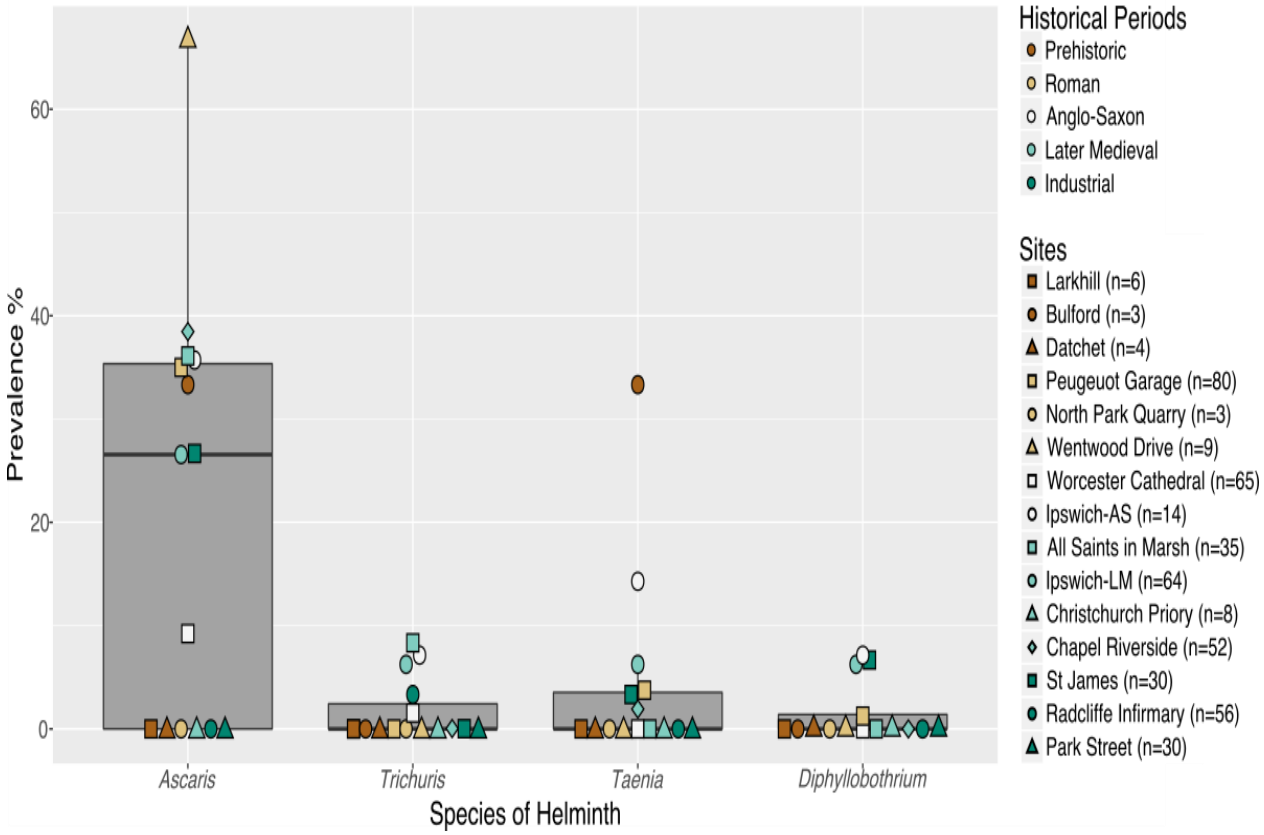


Figure 3.2: A boxplot representing the variation of the prevalence rates of four helminth taxa (Ascaris, Trichuris, Taenia, Diphyllobothrium) for each group in this study that had a sample size of three or over. Point size and colour can be used to distinguish the groups and indicate which time period they represent.

3.4.2 Prevalence Rates Over Time

The groups were also assessed by time period. Our results were consistent with the published literature as there was evidence for helminths for all time periods in our study. Every period, except the Anglo-Saxon period, had a group where no nematodes were detected, however the only period where these groups had a sample size over 10 was the Industrial period. As the recovery of *T. trichiura*, *Taenia* spp. and *D. latum* was low, *Ascaris* spp. was the most robust taxa for exploring change over time. As can be seen in Figure 3.2, there were sites from every time period that had a prevalence rate above the median however, only the Later Medieval and Roman periods had more than one site with a prevalence rate above average. The Kruskal Wallis test was used to assess whether the prevalence rates of *Ascaris* spp. for the skeletal groups were distinct for different time periods, however no significant difference could be detected (chi-squared = 3.36, df = 4, p-value = 0.5).

By combining three modern publications, the average global endemic prevalence of *Ascaris* spp. was calculated to be 19.99% (median=18.10%, range=5.40-49.00%, σ =11.61%) and 14.82% (median=12.70%, range=0.10-38.40%, σ =10.32%) for *T.*

trichiura (based on the data published by Chan et al., 1994; De Silva et al., 2003; Pullan et al., 2014). In historical Britain the average prevalence rates for *Ascaris* spp. was greater (20.52%) and the *T. trichiura* prevalence lower (1.77%) than the modern average, as can be seen in Figure 3.3. Whilst this difference was not significant for *Ascaris* spp. (Welch's t-test, p-values 0.9286), it was for *T. trichiura* (Welch's t-test, p-values 1.0667e-7). Seven of the historical British groups had a prevalence rate of *Ascaris* spp. below the modern average, three of which had a sample size over eight. Of these, two of the groups were from the Industrial period and one from the Anglo-Saxon period.

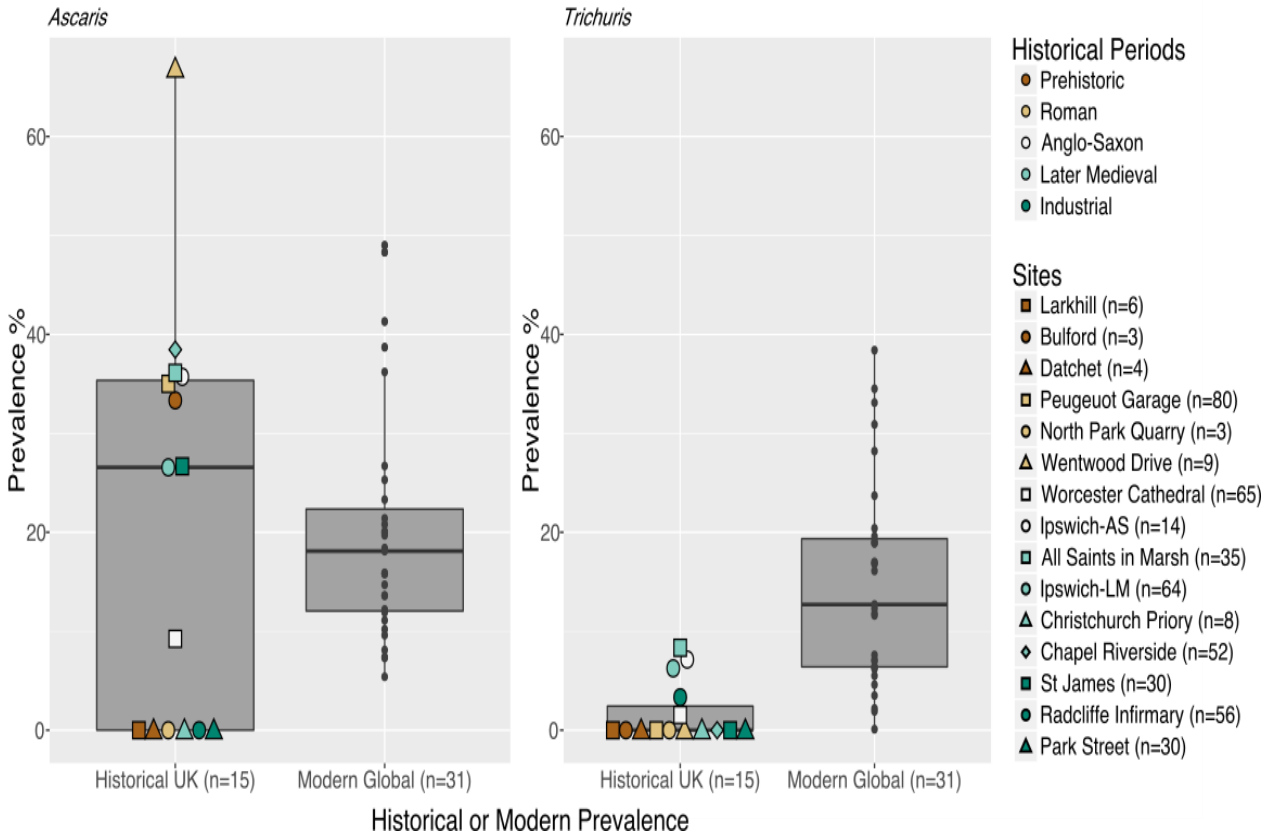


Figure 3.3: Boxplots representing the distribution of prevalence rates for Ascaris spp. (L) and T. trichiura (R) in the archaeological sites in comparison to modern prevalence rates for different geographic regions. The modern data is extracted from Chan et al. (1994); De Silva et al. (2003); Pullan et al. (2014).

The overall prevalence rates per time period was also explored to lessen the effect of differences in group size. In every period, except the Prehistoric, there was a greater prevalence of nematodes (20.63%, range 6.67-38.30%, σ=13.18%) than cestodes (4.38%, range 2.59-6.667%, σ=1.72%) however the prevalence of nematodes predominantly reflected *Ascaris* spp. as it was always more abundant than *T. trichiura* (*Ascaris* spp. average 19.45%, range 6.67-38.29%, σ=14.58%; *T. trichiura* average 1.56%, range 0-4.40%, σ=1.90%). There was a greater proportion of *Taenia* spp.

(3.28%, range 0.86-6.67%, σ=2.12%) recovered than *D. latum* (1.31%, range 0.00-2.52%, σ=0.92%) and this held for every period except the Industrial period.

Prevalence rates for each species per time period is depicted in Figure 3.4. Whilst the prevalence of *T. trichiura*, *Taenia* spp. and *D. latum* remained relatively consistent over time, there was a higher degree of variation in the prevalence of *Ascaris* spp. When comparing the prevalence rate of *Ascaris* spp., the lowest prevalence rates were detected in the Prehistoric period (6.67%) however the Industrial period had a prevalence rate that was nearly as low (6.90%). These periods, along with the Anglo-Saxon Periods (13.92%), had significantly different (p=<0.05) prevalence rates for nematodes in comparison to the heavily infected Roman (38.30%) and Later Medieval Periods (31.45%) (see Table 3.2 for chi-square results).

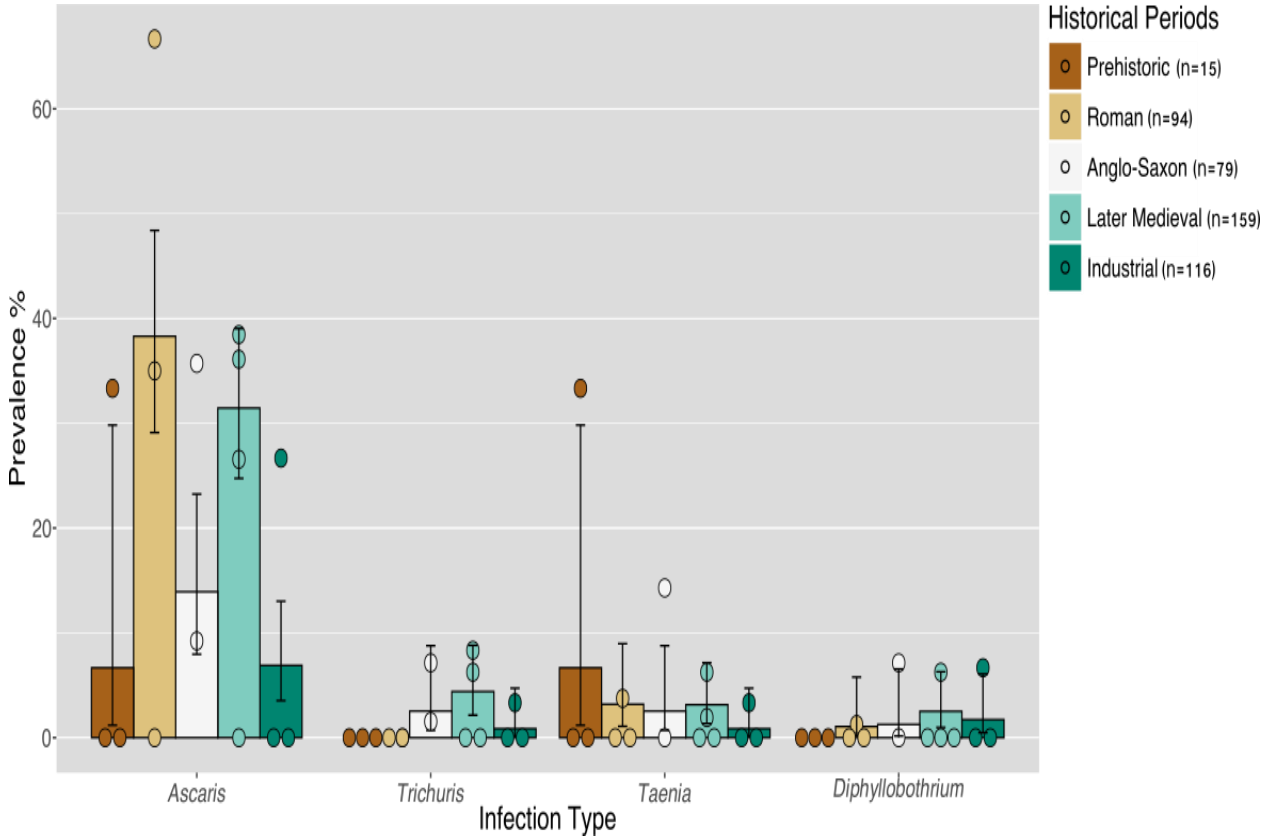


Figure 3.4: A bar chart representing the overall prevalence rates of four helminth taxa (Ascaris, Trichuris, Taenia, Diphyllobothrium) for five different timer periods: Prehistoric, Roman, Anglo-Saxon, Medieval and Industrial. The confidence intervals have been calculated using the Wilson Score interval to a 95% confidence level. The prevalence rates for each group with a sample size of three or more has been laid over the top to see the degree of group variation in comparison to the prevalence rate for the whole period.

	Prehistoric	Roman	Anglo Saxon	Later Medieval
Roman				
Chi-squared	5.719			
DF	1			
Significance level	P = 0.0168			
Anglo Saxon				
Chi-squared	0.590	12.813		
DF	1	1		
Significance level	P = 0.4425	P = 0.0003		
Later Medieval				
Chi-squared	4.039	1.231	8.466	
DF	1	1	1	
Significance level	P = 0.0445	P = 0.2672	P = 0.0036	
Industrial				
Chi-squared	0.001	30.766	2.626	24.200
DF	1	1	1	1
Significance level	P = 0.9737	P < 0.0001	P = 0.1052	P < 0.0001

Table 3.2: Comparison of the differences in the overall prevalence rates using the N-1 Chi-squared test, available through the online MedCalc software.

3.4.3 Prevalence Rates with Age at Death

As can be seen in Table 3.1, 10 of the groups in this study had complimentary osteological data. Nine of the groups had numerical ages assigned to at least a portion of their skeletal individuals, whilst All Saints in the Marsh, York, had individuals categorised either into Adult or Juvenile but no numerical ages were given. Age estimation is precise for juveniles as it is based on tooth development, however the majority of the age estimates come as a range as they are based on wear patterns which does not occur in a consistent manner (White et al., 2011). In our data set the youngest individuals were neonates and the oldest were classified as over 60. Overall, there was a greater proportion of adults (total n=225 (74.50%), n with numerical ages=195) than juveniles (total n=77 (25.50%), n with numerical ages=71) and this held true for every site, except Larkhill. Figure 3.5 shows the age distribution of the samples along with the prevalence of the nematodes and cestodes in different age groups.

Children are more heavily afflicted with soil transmitted helminth infections than adults in modern epidemiological studies, with peak prevalence rates occurring in individuals under the age of 18 (Galvani, 2005). To explore this, we divided our data by the age groups that were reported for the majority of the site reports: 0-5, 6-12, 13-17, 18-25, 26-35, 36-45, 45+. When our historical data set was combined there was no apparent trends in the prevalence rates in association with age (Figure 3.5). The only period where the modern trend occurs is during the Later Medieval period where there is a gradual decline in the prevalence rates between the 0-5 and 13-17 year olds but this

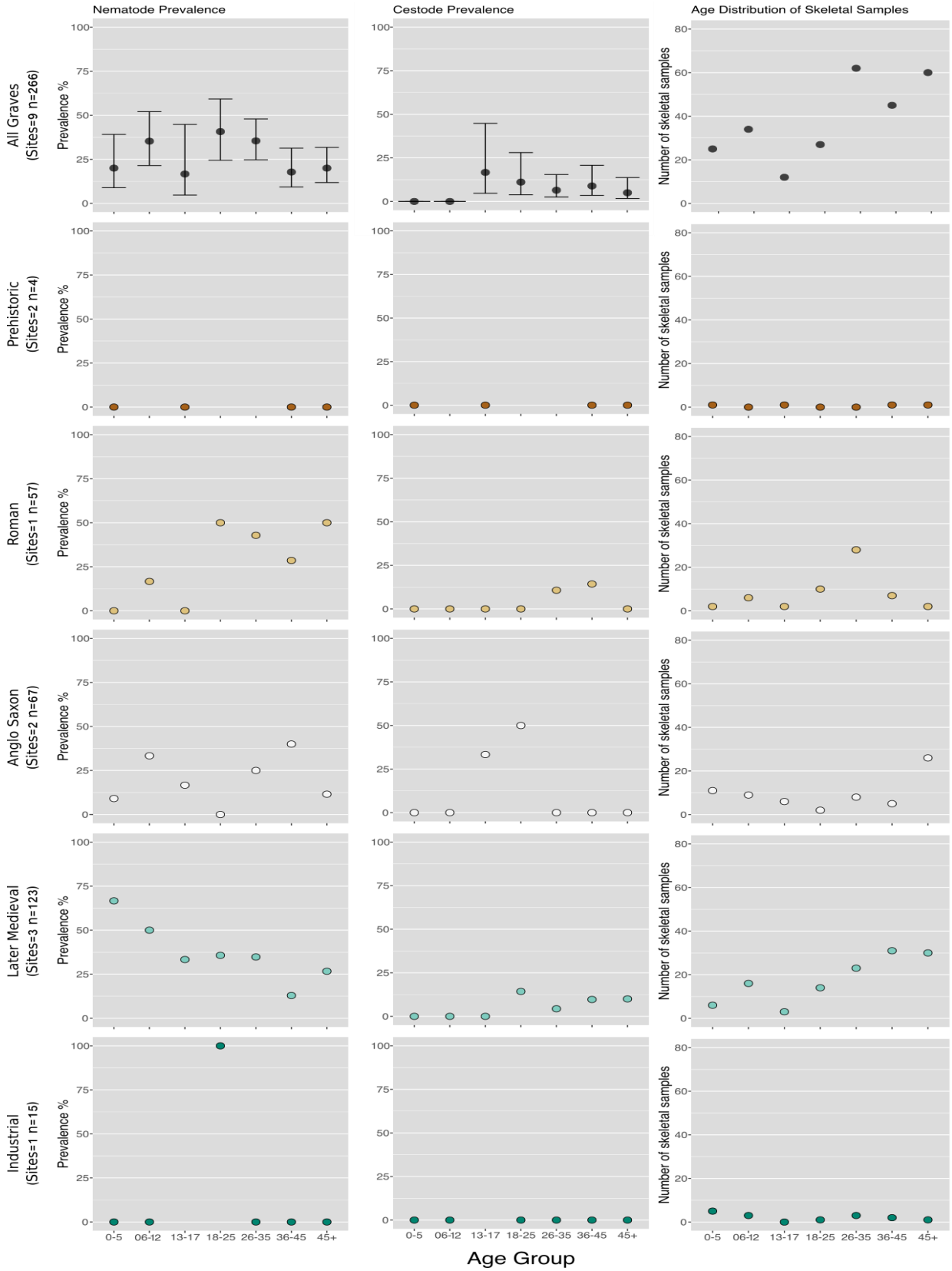


Figure 3.5: Scatter graphs showing the prevalence rates of both the nematodes (L), and cestodes (M) in comparison to the number of samples (R) for different age groups identified using osteological data. The effects of age on prevalence rates can be viewed for the entire dataset and the Prehistoric, Roman, Anglo-Saxon, Later Medieval and Industrial periods. The confidence intervals have been calculated using the Wilson Score interval to a 95% confidence level for the combined data set.

trend was not significant (0-5 to 13-17, Fisher's exact test 2-tailed P= 0.5238). The cestodes *Taenia* spp. and *D. latum* can vary with age if the proportion of meat and fish in the diet also changes with age. There is an absence of these cestodes in children (individuals under the age of 13) throughout our dataset, whilst they are found in adults for the Roman, Anglo-Saxon and Later Medieval periods. This trend is not statistically significant when all the data is combined (0-5 to 13-17, Fisher's exact test 2-tailed P= 0.0991).

3.4.4 Prevalence Rates with Sex

Nine sites in this study provided sex identification for at least a proportion of individuals (33.33-100%), see Table 3.1. Juveniles and incomplete individuals do not have the sex data provided due to the lack of pelvic and cranial indicators used to determine sex (Meindl et al., 1985). 218 individuals (47.08%) were provided with sex data with a slight bias of males (121, 55.50%) over females (97, 44.50%). The prevalence ratio for each helminth taxa and time period is depicted in Figure 3.6. Some of the comparisons could not be included due to a lack of helminths in the sexed individuals but the count values can be viewed in Table 3.3. For any infection there was no significant difference between the two sexes (31.96% female, 24.79% males, Fisher exact two-tailed p value 0.2883) and this held true for each of the parasite types (Fisher exact two-tailed test: *Ascaris* spp. F=24.74% M=18.18% P=0.2475, *T. trichiura* F=1.03% M=4.96% P=0.1349, *Taenia* spp. F=4.12% M=3.31% P=1, *D. latum* F=4.12% M=0.83% P=0.1742). When the data was broken down by time period, there was also no significant difference between the sexes when any infection was considered (Fisher exact two-tailed test: Roman F=46.67% M=35.71% P=0.5279, Anglo-Saxon F=19.05% M=20.00% P=1, Later Medieval F=40.82% M=25.49% P=0.1369).

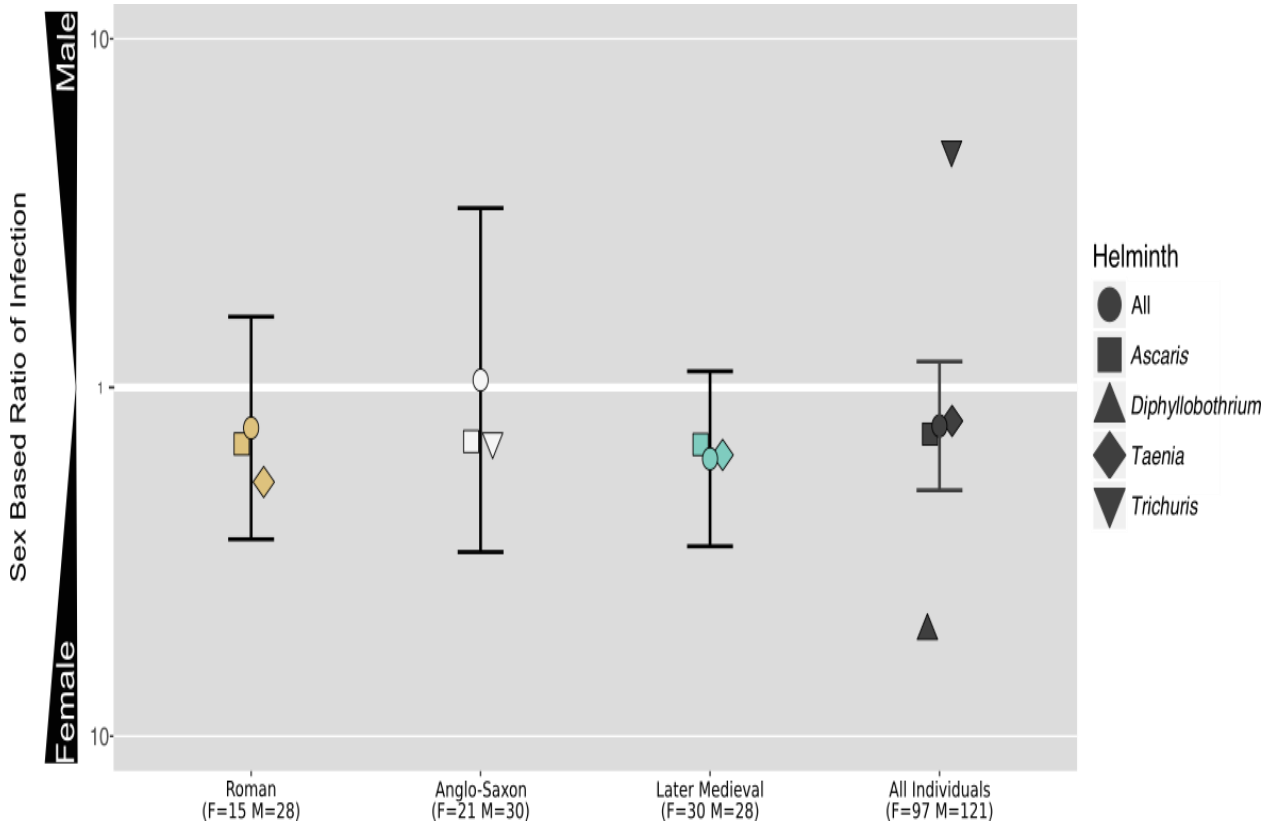


Figure 3.6: A graph depicting the ratio of the prevalence of helminth infections in each biological sex (risk ratio). A value of one indicates that there is an equal proportion of the male population infected to the proportion of females who are infected. A value above 1 indicates that males are more heavily infected, whilst a value below this show that females have the heaviest worm burden. The error bars represent a 95% confidence interval for the risk ratio of any infection.

	Number of Sites	**Individuals**		**Any Infection (n)**		***Ascaris* (n)**		***Trichuris* (n)**		***Taenia* (n)**		***Diphyllobothrium* (n)**	
		F	M	F	M	F	M	F	M	F	M	F	M
Prehistoric	2	2	1	0	0	0	0	0	0	0	0	0	0
Roman	1	15	28	7	10	7	9	0	0	1	1	0	0
Anglo Saxon	2	21	30	4	6	3	3	1	1	0	1	0	1
Medieval	3	49	51	20	13	14	10	0	4	3	2	4	0
Industrial	1	10	11	0	1	0	0	0	1	0	0	0	0
All	9	97	121	31	30	24	22	1	6	4	4	4	1

Table 3.3: A table showing the number of individuals infected according to skeletal sex. Values are counts rather than proportions.

3.5 Discussion

3.5.1 Time period and prevalence rates in Historical Britain

There are three main ways in which parasite prevalence could have changed over time in Britain: a gradual decline, a sudden drop in the modern period or fluctuations between high and low prevalence rates. In historical Britain, the prevalence of parasites appears to have fluctuated over time which suggests that cultural changes were significant enough to affect the prevalence of helminths in the past. Overall the Prehistoric, Anglo-Saxon and Industrial periods all had significantly lower prevalence rates than either the Roman or the Later Medieval periods. At the site level, the Roman and Later Medieval periods both have multiple groups with a prevalence rate above the interquartile range of prevalence rates in modern endemic populations (see Figure 3.3), whilst two of the Industrial groups and the largest group from the Anglo-Saxon period had prevalence rates below the interquartile range. This suggests that these shifts in prevalence rate are large in comparison to modern data, which is built on the assessment of a larger number of individuals from a broader geographical range. Currently, we cannot explore shifts in the prevalence of cestodes over time as the number of eggs recovered were too low to detect any significant differences. Instead, as our results predominantly reflected changes to the prevalence of *Ascaris* spp., the behavioural and environmental shifts to explore are those that have the capacity to disrupt the faecal-oral route of infection.

3.5.1.1 Shifts affecting waste management

Whilst chemotherapy is currently used to control morbidity effects of the STN, policy on WASH practices is being developed as a means to control transmission (Campbell et al., 2018). WASH represents a broad category of behaviours (e.g. hand washing practices, food preparation practices and, use of human waste for fertiliser) and infrastructural changes (e.g. access to clean water, access to latrines and the type of latrines available) which all control the effect of human waste on health. Whilst improvements to WASH are critical for human health, the impact of these improvements is influenced by the type and quality of interventions used, consistency in WASH usage, as well as the behaviour and activity of others in the community (Ásbjörnsdóttir et al., 2017). As summarised in the F-diagram below (Figure 3.7), the routes from faeces to new host is not always direct so the most effective way to prevent transmission is through primary barriers, i.e. hand washing practices and constructing sanitation facilities that restrict faeces contamination into the environment (WHO, 2005). Shifts in hygiene practices could therefore provide one explanation for the shift in prevalence rates detected in historical Britain.

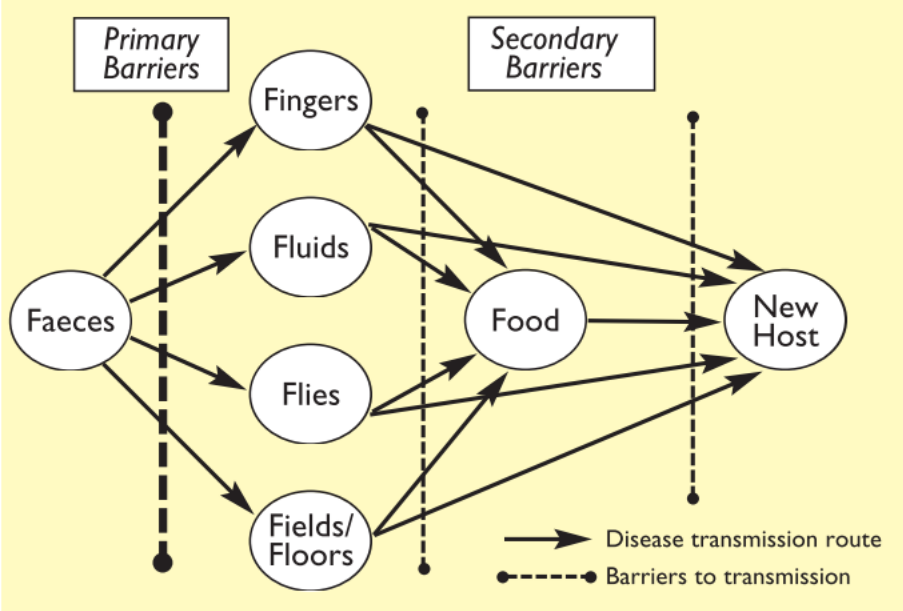


Figure 3.7 An F-diagram from WHO (2005), p10. It illustrates the various routes between faeces and the pathogenic organisms it can contain to new hosts. By visualising routes of infection this way, it highlights the different areas that intervention can be applied.

One shift in prevalence rate between periods in this study where there was a clear difference in hygiene practices was between the Later Medieval period and the Industrial period. The high and late middle ages were periods of increasing urbanization. This led to a rise in the concentration of people and their waste which needed to be kept in check by government restrictions and infrastructural initiatives for waste management (Magnusson, 2013). Unsurprisingly there is also a wealth of skeletal evidence suggesting that living in these urban environments increased the risk of mortality and reduced survivorship in comparison to a rural environment during this period (Walter and Dewitte, 2017), partly due to increased risk of infection. This study found a high prevalence of STN in the Later Medieval period which has also been detected in other studies of European countries. In Chapter 2, prevalence rate for *Ascaris* spp. ranged between 22.0%-42.9% from four Medieval sites (two from Germany, two from Czech Republic) which is similar to the rates detected in the UK.

The Industrial period is also associated with the intensification of urbanization and industrialization, but this ultimately lead to a period of "sanitary reform" in the mid-19th century (Cook, 2001). This reform included the Public Health Act 1848, championing of vaccinations, collection and use of health statistics to prompt public health action, increasing cleanliness and hygiene in hospitals and the construction of sewer networks (Donaldson, 2008). These changes were gradual and initially focused on specific towns, therefore it is likely the eradication of STN was patchy. This study suggest however, that

the impact of these practices were felt rapidly and the dip in the prevalence rates during this time period signifies the beginning of the end of endemic STN in Britain.

Hygiene practices alone however, cannot explain all the fluctuations in prevalence rates in historical Britain. The Roman period is widely regarded as having good sanitation practices including multi-seat latrines with washing facilities, sewer systems, sanitation legislation, regular bathing and aqueducts (Mitchell, 2016). Despite these practices STN have been found in archaeological sites across the Roman Empire (Anastasiou et al., 2018; Bouchet and Le Bailly, 2014; Cox, 2002; Kenward, 2009; Knights et al., 1983; Mitchell, 2016). Our results support this finding and suggests that these hygienic practices had a negligible effect on STN prevalence in historical Britain. As discussed above, partial implementation of WASH practices may not be enough to impact STN prevalence rates, especially in the presence of other mitigating factors such as unclean communal facilities or the use of human faeces as fertiliser (Mitchell, 2016). Further to this, our results suggest a low prevalence rate in the Prehistoric and Anglo-Saxon periods which are not widely regarded as periods with good hygiene practices. This suggests that other factors were at play.

3.5.1.2 Shifts affecting waste production and concentration

As well as cultural changes that improve waste management practices, there are other changes that could affect the concentration of waste in settlement areas. As discussed for the Later Medieval period, the size and concentrations of the population in residential units will influence the build-up of waste in the environment and the risk of coming into contact with infected waste. Sedentism can also increases the capacity for waste to build up which would allow diseases to breed and spread (Rafferty, 1985). This idea is supported by studies which found that modern nomadic groups such as the Penan (Voeks and Sercombe, 2000) and Teda (Cashdan, 2014) suffer from unusually low levels of infection. Fluctuations in population size and settlement types over time might affect the level of exposure to STN and therefore prevalence rates.

The rise of agriculture during the Neolithic had a broad impact on all aspects of human life including prompting communities to become settled and aggregated which resulted in the rise of a host of parasitic and infectious diseases (Larsen, 1995). By the Iron Age, faecal-borne parasites have been found to be ubiquitous in European societies (Reinhard et al., 2013). None of the Prehistoric samples in this study were defined as Palaeolithic but 3/4 of our groups had samples dating from the Neolithic or later. With 15 samples representing this time period, there is a degree of uncertainty whether the prevalence rate established for this period is robust, however currently it suggests that

the rise of STN during this period did not result in a high prevalence rate. Whilst agricultural populations were more settled and living in closer proximity than hunter-gatherer communities, the density of these populations may not have been as high as those in the Roman and Later Medieval periods.

In Britain, the Anglo-Saxon period has less archaeological evidence of settlement structures and power dynamics than many of the other periods of history, as the archaeological evidence is dominated by cemeteries and grave goods (Wright, 2015). This makes interpretation of the living environment harder to infer. Whilst the Roman and Later Medieval periods were defined by large permanent populations clustered around key centres which were vital for trade, administration, religion, and political power (Astill, 2011), the Anglo-Saxon period appears to be characterised by non-nucleated settlement patterns and temporary structures (Hamerow, 1991, 2011, 2012). This suggests that this population could be at a lower risk of STN exposure due to mobile and segregated communities.

3.5.1.3 Shift in capacity for waste management to mitigate waste production

Our data set currently suggests that soil transmitted STN were consistently endemic throughout history in Britain, but the prevalence rates fluctuated over time. The prevalence of soil transmitted STN appeared to dip in periods where the population had dispersed settlement patterns. With a reduction in waste build up from small and temporary settlements, the environment would not be as contaminated with STN ova so the population would be at a lower risk of infection. The population of STN recovered in periods where the human hosts were settled in and around concentrated urban centres. Despite structural and behavioural patterns that could interrupt faecal-oral transmission in the Roman period and the government restrictions and infrastructural initiatives to control waste management in the Medieval periods, these were not enough to have an impact on the STN prevalence rates. It was only with the rise of modern sanitation and the knowledge and drive to implement these practices into the broader population during the 19th century, that hygiene practices became efficient enough to mitigate the effects of waste build up that come with settled and concentrated populations. This study suggests that the effects of the “sanitary reform” on STN prevalence was felt rapidly in the industrial population, so this was the key change that resulted in the eradication of soil transmitted STN as an endemic health problem in Britain.

3.5.2 Prevalence rates at the group level

Whilst the data suggests that there may have been fluctuations in STN prevalence over time, it is important to note that at the group level there was a high

degree of variation. This is not a surprising result as there is variation in prevalence rates between different populations today (Chan et al., 1994; De Silva et al., 2003; Pullan et al., 2014). As well as local differences, in our sample groups this variation could also be influenced by the number of individuals per site (particularly small sites), the population structure of the burial grounds and differential preservation of helminth eggs, although the robust nature of these eggs makes the latter point unlikely (Wharton, 1983). For both the Roman and Anglo-Saxon periods, the majority of samples came from one group so the patterns detected here have the potential to be indicative of site variation. In addition, the sample size for the Prehistoric period was small and therefore further research needs to be undertaken to improve the robustness of the conclusions drawn for this period.

Whilst variation at the site level was detected in the Medieval and Industrial periods, there was a degree of consistency in the prevalence rate between groups. Three groups from the Medieval period had sample sizes ranging between 35-64 with nematode prevalence rates between 29.69-42.86% (1 site outlier n=8, prevalence=0%, this site is also unusual as it is a monastic burial ground) whilst the Industrial period had two groups with sample sizes ranging between 30-56 with nematode prevalence rates between 0-3.33% (1 site outlier n=30, prevalence 26.67%). This suggests that the prevalence rates established in this study for the Later Medieval and Industrial period are not just representative of unusual sites.

3.5.3 Biological risk factors in Historical Britain

As well as assessing prevalence rates over time, changes in risk based on age and sex could also be explored. Research has consistently found that children are at a higher risk of infection and pathology from faecal-orally transmitted helminths than adults, hence why children are the target of the chemotherapy program implemented by WHO (Becker et al., 2018). There is some debate whether this higher prevalence is linked to increased exposure to infection via inadequate sanitation and greater contact to other children, animals and soil, or underdeveloped immune systems and a lack of time to develop acquired immunity (Martins-Melo et al., 2017; Truscott et al., 2014). A number of studies have found that peak prevalence rate occurs prior to the age of 18 for both *Ascaris* spp. and *T. trichiura* (Galvani, 2005). This pattern is not detected in our data. This may suggest eithers behavioural difference in the population of historical Britain so the difference in risk between the ages was less pronounced or, the way the skeletal data was grouped for this study made it impossible to detect significant patterns due to sample sizes being too low.

There is no overall sex associated difference in *Ascaris* spp. and *T. trichiura* infections in modern populations (Brooker et al., 2004) but there are case examples where sex is a risk factor (Gildner et al., 2016; Trang et al., 2007). Whilst sex itself does not increase the risk of helminth infection, behavioural differences that increase risk and are associated with gender roles does. For the periods where risk ratio could be calculated, sex did not significantly impact the prevalence of helminths. This suggests that gender roles in these time periods did not affect risk of infection.

3.6 Conclusion

This study supports previous work that has consistently identified helminth infections in every time period in historical Britain. By applying a wide scale epidemiological approach to single burial samples however, we have established that this consistency in presence was not indicative of a consistency in prevalence. The fluctuations in the prevalence rate over time currently suggests that the concentration of waste in the environment had a more significant impact on the prevalence rate of STN than sanitation practices. That is until the 19th century where a suite of changes in attitudes, behaviour and infrastructure in relation to sanitation and health had a rapid effect on the prevalence rates of STN in historical Britain. In Britain it took around a century between the “sanitary reform” and the eradication of helminths as an endemic medical issue, whilst the holistic approach taken by Japan in the 20th century resulted in the eradication of several major parasitic diseases, including helminths, in 30 years (Kasai et al., 2007).

This result suggests that WASH interventions do need to be wide in terms of scale for there to be an impact on the STN population however, not necessarily as wide as those used in modern non-endemic regions. Considering that both the production and management of waste practices appears to be a key historical factor in the prevalence of STN, future research should use a wide scale epidemiological approach to directly compare urban and rural archaeological sites to establish the influence of regional differences and how that fits into our temporal narrative. The analysis used in this study applied at a finer resolution would mean that specific changes to waste production and management and their impact on STN prevalence could begin to be teased apart.

3.7 Bibliography

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3.8 Supplementary

3.8.1 A brief description of the archaeological information available for each of the sites described in this chapter.

Prehistoric

Group A: 1 sample
Taplow (Newton & Powell site report unpublished) *https://www.wessexarch.co.uk/our-work/taplow-quarry + sent site report*
The site lies ~700 m to the South-East of the centre of Taplow. The majority of the features uncovered date to the Prehistoric period including Neolithic pottery and flints, field systems, postholes, shallow pits, wells, a possible waterhole, a ring ditch and a single with Beaker pottery. There were also two post-medieval pits uncovered from the site. A single grave was recorded at an intersection of a number of ditches of different phases, dated by radiocarbon dating to the Middle-Late Iron Age. It was accompanied by a chalk fragment, large lump of slag (?), a partially complete animal skull, and two large flint nodules.

Group B: 6 samples
Larkhill (Mckinley, 2017; Inés Lopez Doriga pers. comm.)
The Larkhill site is located three kilometres north east of Stonehenge. The site contains evidence of late Bronze Age ditches, Iron Age field systems, lynchets and terraces of probable Romano-British date; and military remains predominantly from World War 1. There is great interest in this region of the prehistoric archaeology as there are a number of prehistoric ceremonial structures in the surrounding landscape. The samples in this study were excavated in two distinct phases. One set of samples were collected from overlying individuals that were in tightly crouched positions and had been badly crushed. These have been identified as prehistoric (radiocarbon dated to Middle/Late Bronze Age). Two came from adjacent graves (consisting of an adult female and a 15-17 year old male) which had been disturbed, whilst the third was a neonate found in a pit.

Group C: 3 samples
Bulford (Harding, 2016; Inés Lopez Doriga pers. comm.)
Bulford is a village about 3 miles East of Larkhill and around 2 km to the east of Stonehenge. The site consists of two ring ditches and a number of Late Neolithic pits. Excavation suggests that the ring ditches represented Bronze Age re-use of Neolithic hengiform enclosures. The site also has uncovered two Bronze Age round barrows and an Anglo-Saxon cemetery with 150 graves and an abundance of funerary goods. There is also some evidence of World War 1 practice trenches and World War 2 anti-tank firing ranges.

Group D: 4 samples
Datchet (Inés Lopez Doriga pers. comm.)
Datchet is a village in the Borough of Windsor and Maidenhead. Research in this region has produced finds from multiple time periods. The earliest include Late Glacial worked flints and a Mesolithic worked flint Axe. There is also evidence of Neolithic material remains, Bronze Age field systems with Deverel Rimbury pottery, Romano-British field system and a lost medieval trackway. The greatest amount of archaeological material comes from the Bronze Age period as organic material has been well preserved at the bottom of waterholes and within ditches.

Group E: 1 sample
Vale Crematorium (Capps & Wakeham 2018)
Vale Crematorium is at Fladbury Cross, Worcestershire. There are two key features that define this site: a Penannular enclosure and a Ring Gully. The site also included 3 smaller linear features and a medieval ditch. The Penannular enclosure was relatively substantial and the fill sequence suggests that it naturally silted up but also contained a small quantity of Early–Middle Iron Age pottery. On the western side of this feature, a single crouched inhumation was uncovered. It was uncovered with an accompanying copper alloy object although there are no further descriptions of what this object was.

Roman

Group F: 1 sample

SSSM (Wessex Archaeology, 2016; Inés Lopez Doriga pers. comm.)

SSSM refers to excavations carried out in support of the construction of a water pipeline from Barrow Gurney, North Somerset, to Cheddar, Somerset. Whilst evaluation trenches were carried out along the route, the village of Churchill, Somerset, was identified as a region of interest and the basis of a full excavation. The site is a Romano-British settlement including an enclosure, burials, associated boundary ditches and a large pit which may have been a quarry. There was also Romano-British pottery of moderate quantity which was used to date the site and as the basis for assigning the site as a settlement. We received one sample from this site which was from a slumped or face down burial. This burial is also believed to be Romano-British is date due to the presence of hobnail boots and pottery.

Group H:80 samples

Peugeot Garage (Enid Allison pers.comm.)

The Peugeot Garage site is located in Canterbury. It is currently located at the edge of the city centre opposite the city walls. The site consists of 194 inhumations with a clear distinction for the elite burials, located to the East of the main burial area. One set of elite burials are within a chapel, but both sub-sets are identified as higher status due to the layout of the graves, the depth of the burials, and the high number of grave goods. The soil samples analysed here are from the lower status burials. During the early Roman period, this site would have lay outside the Roman town on the principal route between Canterbury and Dover. Residual pottery identified in the backfill suggests that the majority of the graves date between the 2nd and 3rd centuries, whilst a handful date to the 1st, 4th and 5th centuries. Unfortunately, a third of the graves remain undated due to a lack of finds but these are also presumed to be Roman in date.

Group I: 3 samples

North Park Quarry

http://archaeologydataservice.ac.uk/archives/view/northpark_he_2016/

Sand quarry to the south of North Downs. When the topsoil was removed a number of features from a range of time periods was uncovered. These included Mesolithic, Neolithic, Bronze Age and Iron Age features, predominantly consisting of pits. Whilst the Roman period is only represented by stray finds, Saxon and Early Medieval activity was indicated by a ditches, pits, post holes and a probable waterhole.

Group J: 9 samples

Wentwood Drive (Holt site report unpublished)

The site is approximately 1.5 km north-west of Bleadon and represent a Late Iron Age and Romano-British population. From the Late Iron Age period there is a ring ditch, postholes, pits and inhumation burials. The Romano-British feature consists of multiple small terraces cut into the hillside, pits and inhumation burials within both distinct grave cuts and within reused refuse storage and quarry pits. After the Romano-British period there is no further evidence of activity and the structures and terraces fall to ruin. The samples used in this study date to the Romano-British period.

Anglo-Saxon

Group K: 65 samples

Worcester Cathedral (Guy, 2010; Waldron, 2010)

The skeletal samples come from an excavation under the Norman Chapter House within Worcester Cathedral. The limited number of grave goods and the alignment of the bodies suggested that this was a late Anglo-Saxon Christian cemetery. The mixture of ages and genders, amount of intercutting and knowledge of the construction of the earlier cathedrals; suggests that this was a lay cemetery. It is suggested that it was originally

located outside the cathedral walls and was used for a prolonged time prior to the Conquest period. The designation of a lay cemetery is further supported by the lack of charcoal burials, which at the time was commonly used for high status urban ministers. Whilst there was some evidence of wooden coffins and fabric shrouds with some of the bodies, the skeletons themselves were generally poorly preserved and discoloration affected about 1/5 of the skeletons. Whilst there are some grave goods (including a padlock and key, a glass bead, a lead cross, a pin and coins) there is no record to identify which individuals were associated with these finds. In terms of pathology and achieved heights, the cemetery was an average representation for this time period.

Anglo-Saxon to Later Medieval

Group L: Anglo-Saxon 14 samples, Later Medieval 64 samples

Ipswich (Farber & Lee-Thorpe in prep, Rebecca Nicholson pers.comm.)

Ipswich started as a small trading settlement in the early 7th century before becoming a flourishing town ideally situated to trade with Germany. The soil samples come from the lost cemetery of St Augustine's which dates from the late Saxon period to the dissolution in the 1530's. The graves used in this study have been classified into three time periods: 9th-10th, 11th-12th and 12th-15th. The first phase refers to the late Saxon settlement pre-dating a stone church, the second is during the church construction and the third is the last phase of use before the church was abandoned, probably in 1499. The site has a total 1140 burials however this research focused on a smaller sub-set of samples some of which also had Carbon, Nitrogen, Oxygen and Strontium analysis undertaken on them. The bone collagen samples were selected based on the date, location within the burial ground, age and sex. The tooth analysis however was aimed at identifying 'non-local' individuals and so a small sub-set of individuals with unusual osteological features were selected.

Later Medieval

Group G: Roman 1 sample, Later Medieval 35

All Saints in the Marsh, Peasholme, former Haymarket (Reeves 2016)

Land formerly occupied by the Peasholme Centre hostel buildings is a site within York close to the Rive Foss and within the city walls. It is one of the largest excavations undertaken in the center of York. Our samples are from the excavation is 2012. There has been a wealth of evidence from a range of time periods including a Roman *canabae*, trade during the Anglo-Scandinavian period, the Medieval church of All Saints and neighbouring Priests' house, domestic construction beginning between 1810-1815, and evidence of demolition of 'slum' houses in the 1930's. All but one of the skeletal samples date from the Medieval period as part of the graveyard of All Saints in the Marsh. The one exception was overlain by a church wall and has subsequently been identified as Roman. The church was believed to be founded around the time of the Norman conquest and served the relatively poor parishes before being deconsecrated in 1586. Whilst there were 507 samples excavated, we only have a sub-sample of 35 samples. These skeletons had not been cleaned and therefore pelvic soil samples adhering to the skeletons could still be collected. The post excavation analysis is still incomplete and therefore the osteological analysis from this site is limited to within site observations.

Group M: 8 samples

Christchurch Priory visitors Centre (Dinwiddy Site report 2018)

Christchurch is a coastal town in Dorset. The site, whilst containing Mesolithic and Early Neolithic worked flint and a 17th Century Civil war ditch, consists mostly of finds associated with a Medieval Monastic Cemetery. The majority of the burials at this site are of older males and therefore it is suggested that the cemetery was largely reserved for the brethren. The burials consist of a mixture of inhumations, burials in shrouds, burials in coffins and burial in stone-lined graves. In 1094 the church was built, in 1150 it was refounded and then eventually dissolved in 1539. Whilst the majority of samples

received are believed to date to when the church was active, two samples (241 and 339) come from a collection that are believed to pre-date the church as they are truncated by the first identifiable phase of the monastic cemetery. These samples have been classified as Early Medieval. As the site report references the Saxon period specifically in regards to other archaeological sites, in this study it is presumed that these samples are not Anglo Saxon and fall within our Later Medieval classification.

Group N:52 samples
Chapel Riverside (Andrews and Orczewski 2017)
Chapel Riverside is a site in Southampton that during the Anglo-Saxon period, lay on the outskirts of a town called Hamwic. Evidence of this occupation comprised of pits, a ditch, a post built structure and potentially a collection of burials. These are still to be dated and have not contributed to the samples analysed in this study. In addition there were substantial remains from a post-Medieval tide mill, a miller's house and an associated mill pond. The samples for this study however come from the Medieval burials from the cemetery of the Holy Trinity chapel. The site was used at least from the beginning of the 13th century until the mid 16th century. The burials represent a 'normal' population in terms of age and sex. In total 122 individual graves were identified along with a number of features related to the construction and use of the chapel.

Industrial
Group O: 30 samples
St James Burial Ground (Don Walker pers.comm.)
This site is in London and was the overflow cemetery for St James Piccadilly. It opened in 1788 and closed in the mid-19th century. The cemetery has 4 distinct regions with increasing status in the regions closer to the church. Samples analysed in this study were from a preliminary assessment and sampling was targeted to try and represent the range of the site. Samples/Skeletons 1069, 3081, 3089, 3091 and 3095 had predominantly vegetation/mossy substrate found within the pelvic cavity. The site osteologists suspect that these were plant material placed into the coffin prior to closing (as floral tributes or to soak up body fluids). Due to the good conditions and the excellent preservation of some of the coffins, there was little soil intrusion.

Group P: 56 samples
Radcliffe Infirmary Burial Ground (site report and nhs website https://www.ouh.nhs.uk/hospitals/jr/radcliffe-infirmary.aspx (accessed 21/11/2018)
The Radcliffe Infirmary was a teaching hospital that was established, with its own burial ground, in 1770 within the Parish of St Giles. The hospital was heavily used despite the restrictions on admissions including people suffering from infectious diseases, pregnant women, the mentally ill and children under seven (except for major operations). As the hospital was run by donations, individuals could only be admitted on a donor's 'turn' and this practice continued until 1884. The burial register and the excavated skeletons show that a greater number of males died within the hospital and that many of those interred were not from Oxford city but were from elsewhere in the country. All burials (except one in 1771) took place between 1815 and 1855 when the burial ground was closed.

Group Q: 30 samples
Park street Burial Ground (Don Walker pers.comm.)
Park Street was an overflow burial ground for St Martin's Church during a period of rapid expansion of the population. It was closed in 1880 and made into a public park instead. Currently zoning has not been identified but it may be that the vast majority of the burial population were relatively poor. Samples analysed in this study were from a preliminary assessment and sampling was targeted to try and represent the range of the site.

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3.8.2 Exploration of other information within the dataset

What was the impact of helminths on health?

Helminth infection does not necessarily lead to noticeable pathology, however in children it can cause retardation to physical and mental development due to helminth reducing the nutrition available for the host. In cases where children are already malnourished, the medical implications are greater. Whilst malnourishment can manifest itself in clear osteological changes (rickets, scurvy, stunting) it can also be inferred indirectly from generic indicators of stress such as cribra orbitalia and enamel hypoplasia. Under 18's with pathological data were selected for an assessment of the correlation between helminth infection at death and skeletal indicators of stress. This age group were selected because in adults there may be quite a large temporal distance between the event causing the skeletal changes and helminth infection. 13 juveniles in our sample set had a helminth infection and records of skeletal pathology. Of these, 8 had skeletal indicators of stress (cribra orbitalia, enamel hypoplasia and /or scurvy). 11 out of 22 juveniles with records of skeletal pathology and no evidence of helminth infection had skeletal indicators of stress. The low numbers make it difficult to assess whether there is a significant difference between these 2 groups however with future research we can begin to assess the correlation between these two indicators of pathology, even if we can not guarantee causality.

Palaeoparasitology combined with isotopic analysis

Isotopic analysis was undertaken on a sub-sample of the skeletal remains from Ipswich, Stoke Quay by Eleanor Farber. Nitrogen analysis of bone collagen was used to indicate individuals with a high trophic level diet (above 13‰). These individuals were less likely to be infected with parasites, in particular *Ascaris* spp.. In addition, three individuals were

identified as non-local from Strontium and Oxygen isotopic analysis of tooth enamel. These three samples had parasite eggs present in the pelvic sediment sample so there is the potential that with genetic analysis, we may be able to identify whether these parasites have a unique haplotype that would provide further support to their identification of being non-local individuals.

3.9 Permission from co-authors.
I hereby give permission to Hannah F Ryan to use our joint work as contribution towards her D.Phil. thesis to be submitted for examination at Oxford University.

I confirm that to the best of my knowledge, the author contribution statement below is accurate and Hannah F Ryan's contribution towards the work is greater or equal than that of any other co- author.

Chapter author contribution statement: H.R., P.F., A.S. and G.L. designed the research; H.R. and P.F. collected samples; B.R., R.N., L.L., E.F., C.G., T.W., E.A. and I.L.D. provided samples and archaeological context; E.F performed isotopic analysis; H.R. and P.F. performed microscopic analysis, H.R. produced statistics and graphs from the count data with input from P.F. and A.S.; A.S. and G.L. secured funding for the project; H.R. wrote the initial draft of the manuscript with input from P.F., A.S. and G.L

Date: 17/4/2019
Name Greger Larson



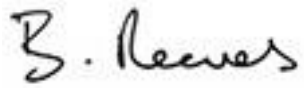
Date: 26/4/2019
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Date: 3/4/2019
Name: Ben Reeves



Date:29/3/2019
Name: Enid Allison



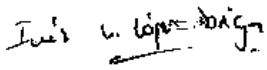
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Name: Chris Guy



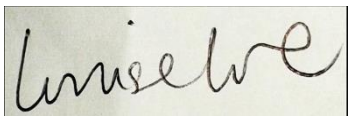
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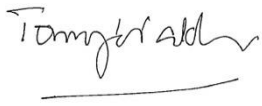
Date:10/4/2019
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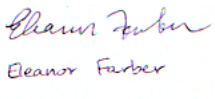
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Chapter Four: The utility of helminths from fluid collections for the construction of 'near modern' reference genomes from non-endemic countries

4.1 Abstract

Using proxies to infer human migration is a well-established tool in archaeology. The success of these proxies is reliant on a close relationship of the proxy to human behaviour and the extent to which it can migrate independently. When pathogenic organisms are used as a proxy they also provide historical evidence of how diseases are spread. Helminths have the potential to be an excellent proxy for human behaviour but a major limitation of the approach is that helminths are no longer endemic in various regions of interest. A modern genetic reference database therefore cannot be completed which will limit the capacity to draw robust conclusions on phylogeography. Museum fluid collections house helminths from geographic locations that are currently free from infection and therefore they could be used to build a 'near modern' genetic database. Samples such as these often go through a process of formalin fixation which has the potential to make genetic analysis problematic. In order to establish whether a widescale analysis of museum samples could contribute to the study of palaeoparasitology, we conducted a pilot study to assess the quantity and quality of genetic data retrievable. One of the seven samples analysed contained endogenous DNA that could be used to identify the genus of the specimen, but further analysis on the sample was limited by the low number of sequences recovered and the variability of loci available in the published reference genomes. For now, the destruction of museum samples is iniquitous with the quantity of data that can be retrieved. When the database of helminth species genomes is improved however, the application of enrichment and bioinformatic approaches will be more powerful and museum samples will have the capacity to be a useful resource for palaeoparasitology.

4.2 Introduction

The phylogeographic analysis of parasites is a relatively recent field of research, which has the potential to be utilised to also infer the phylogeography of the parasite's hosts (Morand, 2012). Bioproxies are regularly used to trace human migration, the most notable of which is *Helicobacter pylori* where the genotyping of this species is a close reflection of genotyping of humans. *H. pylori* is believed to have been part of the human microbiome prior to human expansion out of Africa as the diversification of strains reflect one or more of the major out-of-Africa migrations, hence the degree of similarity between host and parasite (Dominguez-Bello and Blaser, 2011). Most studies in this area focus on viral or bacterial organisms, however, few studies have assessed regional genetic

variation of helminths. A strong regional genetic structure was found on a global scale for the ruminant Nematode *Haemonchus contortus* (Troell et al., 2006) and a gradient from a western to an eastern haplotype in Europe was identified in *Trichuris muris* along with an unique 'Canary Island' haplotype (Callejón et al., 2010). These geographical signals have not however, been found in every helminth that has been studied possibly due to the species' life cycle (such as the effect of multiple host species) or the effect of modern human influence, in particular the decrease of regional signals from increasing globalisation (see Zarlenga et al., 2014).

With a targeted assessment of their modern genetic variation, helminths could be used widely to understand host migration and the spread of disease, both in modern and archaeological settings. Helminths can be found in the archaeological record in both latrines and pelvic soil samples from single graves (Côté and Le Bailly, 2017). Whilst these parasites have been analysed within archaeology to understand various aspects of human behaviour, published work has only begun to scratch the surface of their ability to elucidate past human migration. Flammer et al. (2018) for example, detected connectivity between two Medieval trading ports using a distinct historic clade of the ITS1 gene of *Trichuris trichiura* however, as it has only been detected in these two sites questions remain around the distribution and apparent extinction of that clade.

There are multiple advantages of using phylogeographical analysis of helminths as a proxy for historical human migration. First, the eggs are highly resistant to diagenesis. Modern *Ascaris* spp. eggs are known to survive strong acids, bases, oxidants, reductants, protein-disrupting agents, and surface-active agents (Oh et al., 2016). Not only this, these eggs have longevity to the extent that they can still be identifiable by microscopy in samples dating back to 240 million years BP (Araújo et al., 2015). The survival of aDNA in skeletal remains is dependent on both the age of the sample and the climate (Smith et al., 2001), therefore there is a bias in the number of skeletal samples possessing aDNA at the higher latitudes. No studies have been undertaken on the effect of age and climate on the preservation of helminth eggs and ancient DNA, so it may be possible that helminth eggs survive and retain genetic information in regions where skeletal remains are highly degraded. They may therefore, have the capacity to infer human migration in regions where other bioproxies fail.

Secondly, the life cycle of some of the helminth species limits their migration only to the movement of humans. There are two key species that fulfil this criterion: *Ascaris lumbricoides* and *T. trichiura.* These two species are transmitted by the fecal-oral route (no intermediate host) and both are commonly found in archaeological contexts. In

addition to this, the two species require ingestion of the eggs by human hosts for their full development and thus cannot migrate independently (CDC, 2013). Whilst there are other species of helminth that infect humans, these either require intermediate hosts which would introduce another mode of migration or are not commonly detected in archaeological sediments. Finally, as the generation time is shorter and the level of fecundity is higher in helminths relative to humans, one would expect greater genetic variance in helminths (Whiteman and Parker, 2005). These factors suggest that genetic analysis of helminths from archaeological contexts would be suitable for fine scaled reconstruction of human migration.

Interrogating historical dispersal however, requires the establishment of a baseline knowledge of diversity within and between geographic regions for accurate interpretation. The more data, the more direct comparisons can be made between different regions and time periods to identify the development, continuity or replacement of populations. Whilst ancient DNA is vital for inferences on deep history, it is also affected by degradation and limited by availability. A complete modern reference database covering a wide range of regions can therefore provide context for the interpretation of ancient studies. In isolation, ancient studies can be used to identify genetic links between sites, but without the support of a modern database it can be difficult to distinguish true cases of dispersal rather than variation, trace directionality of movement or identify novel ancient haplotypes. Combining modern and ancient DNA completes the databases and therefore is the holistic approach for understanding the dispersal and evolution of helminths (Parducci and Bennett, 2017).

The lack of genetic research into the historical spread of helminths so far may be due to the limited nature of the modern genetic database both in terms of geography and gene regions. This problem is amplified by the fact that helminths are no longer endemic globally. In regions such as Europe, North America and Japan, there are now only a handful of helminth infection cases (Pullan et al., 2014), most of which are from travellers to high risk countries (Tomaso et al., 2001) or zoonotic cross over (Nejsum et al., 2005). Whilst there are still cases of helminths in low income eastern European countries (Hotez and Gurwith, 2011), it is hard to tell whether the helminths here would have the same haplotypic diversity as those that were once found across the continent. To improve our capacity to utilise helminths for assessing migration in the past, we need to improve the modern reference database both in terms of depth and breadth, including regions which are no longer endemic. Analysis of museum samples is one of the possible ways to fill in this gap in the database. Morphological analyses on helminths in fluid collections have

already provided insight into species identification, disease diagnosis, epidemiology, taxonomy, biogeography and phylogeny so successful analysis using biomolecular approaches has a high degree of potential (Herniou et al., 1998).

In the 19th century, academics began to recognise the utility of fluid collections for teaching and research for both zoological and medical studies. A wide variety of ethanol-based solutions have historically been used for storage but, today Industrial Methylated Spirit (IMS) is the main storage fluid used by museums. Ethanol-based solutions are excellent for preserving DNA, but most samples are fixed for storage, most commonly with formalin (Schander and Halanych, 2003). Formaldehyde, the main component of formalin, causes crosslinking between proteins and nucleic acids by methylene bridges as well as induces methylol modification of nucleobases (Zimmermann et al., 2008). The degree of fixation, fragmentation and damage to DNA appears to be dependent on concentration, the length of exposure, and most importantly the pH and whether the formalin was buffered during the fixation process (Herniou et al., 1998). The problem is that there are not always records of how samples were fixed, or whether fixation has occurred at all. Further, whilst the current storage solution can be determined, there is no guarantee that this was the original storage solution or that the past practice of 'recycling' solution between different specimen is not applicable for the sample (Mark Carnall pers comm).

Due to the high potential of fluid collections to resolve ecological and evolutionary questions, multiple researchers have developed techniques for working with these types of samples. Many of the papers discussing formalin fixation effects on DNA retrieval had samples that had either been frozen or embedded in paraffin or celloidin (Gilbert et al., 2007; Niland et al., 2012). There are a handful however, that have attempted extractions from museum fluid collections from lizards (Hykin et al., 2015), snakes (Ruane and Austin, 2017), human medical samples (Paireder et al., 2013) and helminths (Li et al., 2000; Snábel et al., 2012). The latter two papers were on samples that were 24 and 40 years old, respectively. The general approach for tackling formalin fixed samples is to include a heating step to reverse the crosslinking, however, this requires some care as the heat can also damage any DNA present in the sample. Research suggests the optimum heating step depends on the organism and the fixation process (Schander and Halanych, 2003) but records of the fixation process are often missing for museum samples. Whilst all the published papers cited successfully extracting endogenous DNA, the quality and quantity of the DNA was not always high enough to resolve their research

aims. In addition to this, where multiple samples were used, age of the sample was an important determinant of the success of the extraction.

Despite the issues associated with museum samples, they still have an enormous potential to fill the gaps in the databases and to lay the foundations of a 'near modern' genetic database for helminths in non-endemic areas. Here, we assess the feasibility of utilising museum fluid collections for the construction of a 'near modern' comparison database. The number of successful extractions combined with the quality of the data retrieved, will indicate whether the damage to museum collections that would occur with a wide scale analysis would be counterbalanced by the valuable contribution gained in the field of palaeoparasitology.

4.3 Materials and Methods

4.3.1 Material and Sampling

Seven samples from Oxford's Natural History Museum were selected to test the feasibility of extracting DNA. The samples were selected to include a range of species, and ages, as far as the written records were available, see Table 4.1. Two of these samples have recorded dates, 1886 (MW3) and 1917 (MW8) making these samples more than double the age of the helminths that have been previously studied. Jars which had multiple worms in them were actively selected, under the assumption that the worms came from the same host and time. In these cases, the worms that were not sampled could be retained for future research. To explore the significance of visual cues for assessing the quality of the DNA preserved in the sample, we selected one specimen (MW6) where the majority of storage solution had evaporated as can be seen in Figure 4.1. A portable density meter was used during the sample selection process to assess whether the samples were being stored in the optimal concentration of ethanol (70-80%). This was used as an initial indicator of the preservation of the samples. Whilst there were no details of the storage solution of these samples, there was an assumption that they had been fixed in formalin in the past.

A sub-sampling strategy was developed to minimize the destruction of the museum samples and maximize the retention of the morphologically informative regions. Factors that are utilized to distinguish helminths by morphology include body length and body diameter, but the anterior and posterior regions are the most morphologically informative (Bhadury et al., 2008; Mattiucci et al., 2014). To preserve the distal ends, a square of skin from the central portion of the worm was targeted, see Figure 4.2. This strategy meant the head, tail, internal anatomy and sex organs remained intact. One further advantage of this sampling approach is it limits the quantity of the parasite's host

Extraction number	Museum ID number	Species Identification	Concentration of EtOH	Tissue weight
MW1	OUMNH.ZC-L10 6.	*Ascaris lumbricoides*- no date Incubation One- 2 hours	73%	300mg
MW2	OUMNH.ZC-L10 6.	*Ascaris lumbricoides*- no date Incubation Two- 24.5 hours	73%	300mg
MW3	OUMNH.ZC-2608	*Macracanthorhynchus hirudinaceus*- 1886	73%	95 mg
MW4	OUMNH.ZC-2775 (large jar)	*Taenia saginata*- no date	72%	93 mg
MW5	OUMNH.ZC-2775 (small jar)	*Taenia saginata*- no date	73%	100mg
MW6	OUMNH.ZC-4770	*Fasciola hepatica*- no date	5.2%	100mg
MW7	OUMNH.ZC-D151	*Ascaris lumbricoides*- no date	70%	48mg
MW8	OUMNH.ZC-5363	*Ascaris lumbricoides*- 1917	71%	57mg

Table 4.1: The details of the 8 extracts from 7 worms collected from Oxford's Natural History Museum.





Left- Figure 4.1: An image of the storage of MW6 and the clear evaporation of the storage solution.
Right- Figure 4.2: An image of the subsampling strategy on MW8.
Images are copyright of Oxford University Museum of Natural History.

DNA being incorporated into the extract (Fierst and Murdock, 2017). We aimed to collect at least 50 mg of tissue.

4.3.2 Methodology development

This study's methodology was developed by testing two different protocols on sample "L10.6" (MW1-MW2) as the sub-sample weight was high enough to attempt multiple rounds of extraction. After sub-sampling the tissue and breaking it down in a beadbeater, DNA was extracted following the kit instructions from both the Qiagen DNeasy blood and tissue kit and Qiagen DNeasy mericon food kit ensuring proteinase k was added prior to either heating stage. The two extracts were further divided so they could undergo two incubation processes based off other published approaches. The first had an incubation period of 56°C for 1 hour followed by 90°C for 1 hour (based on Paireder et al., 2013). The second protocol had an incubation period of 90°C for 30 minutes followed by incubation at 55°C for 24 hours (adapted from Ruane and Austin, 2017). The food kit was excluded from further tests as the heating steps caused a reaction in the lysis buffer resulting in the loss of the sample.

For the successful extractions, the extract was appraised using a Qubit fluorometer (Thermo Fischer Scientific) and targeted PCR (utilising the *Ascaris*-specific primers in Table 4.2) followed by electrophoresis and tapestation analysis. Neither of these extraction methods produced positive results (see Supplementary 4.8.1 for details). Whilst these rounds of extractions were intended to assess the suitability of the two methods, there was no guarantee that the sample "L10. 6" retained endogenous DNA. Therefore, the shortest extraction method (Incubation 1) was selected for the remaining museum samples to minimise heat-induced aDNA damage. Shotgunning

Target Name	Bases	Sequence	Length of product
Taenia_Cote_COX1_F	22	GAGGTTTTAGGTTCWTATGGTT	
Taenia_Cote_COX1_R	22	CATTATGAGAAGCYACAGGACT	73 bp
Taenia_Cote_CytB_F	25	ATATGGCTCGTGCTTTGTATTATTC	
Taenia_Cote_CytB_R	27	AGGTAATATATATCCWGTAAAAGCCTC	113 bp
Ascaris_Cote_CytB_F	23	GCCAAAGCACCATCATTAGAATA	
Ascaris_Cote_CytB_R	22	GGTATGGTTTTGGGTTTTCAGA	74 bp
Ascaris_Cote_NAD1_F	25	GCGTATTGGYCCTAATAAGGTTAGT	
Ascaris_Cote_NAD1_R	21	CCGAAGAATTCARAGGAGTCA	104 bp
Mhirudinaceus_ND1_F	20	TGGTGGCGATTGACTTACCC	
Mhirudinaceus_ND1_R	20	ACAACAGGCAAAAACACGCA	77bp
Mhirudinaceus_ND6_F	20	GGGAGGTGGGCGATGTTATT	
Mhirudinaceus_ND6_R	20	CTAACGACCCCCACGTGTTT	94bp
Mhirudinaceus_CytB_F	20	TTGTTTAGCGTGCCTTGTCG	
Mhirudinaceus_CytB_R	20	TAACACTCACACTGCTGCCC	78bp

Table 4.2: The details of the primers used in this study. Any target with Cote in the name were designed and developed in Côté et al. (2016) whilst primer beginning with Mhirudinaceus were designed in this study.

sequencing and the following data analysis were only applied to MW1 to ensure consistency in the methodology when comparing all seven samples.

4.3.3 Methodology

The six remaining museum samples were bead beat (Mini BeadBeater 8, Biospec Inc, USA) with 1mm glass beads in a solution with 80 μl of PBS buffer for a minimum of three minutes, or until the tissue was disrupted. The samples were then centrifuged for 10 minutes and then the supernatant was removed. 180 μl of ATL buffer and 20 μl of proteinase k was then added to the pellet and mixed by vortexing. The tissue was then incubated at 56 °C for 1 hour and then at 90 °C for a further hour. For the first hour, the sample was kept in rotation in a tube rotator. After cooling to room temperature, 2 μl of RNase (100 mg/ml) was added and left to incubate for two minutes. 200 μl of AL buffer was added, followed by vortexing, and then 200 μl of ethanol was added and then vortexed. After brief centrifugation, the solution was loaded onto a MinElute Column. The tube was then centrifuged at 6000 g for one minute. Then two wash steps were run before the column was eluted twice to produce 100 μl of extract. The extracts were run through a qubit and tapestation to assess the quality of the extracts.

All samples underwent shotgun sequencing which consisted of a library building stage following the Meyer & Kircher protocol (2010), qPCR to assess the number of cycles required for amplification, PCR, quantification by tapestation then pooling and cleaning for HiSeq sequencing. In addition, 5 further samples were selected (in addition to MW1) for amplification using a targeted PCR approach. The targets were selected based on the museum identifications. The *Ascaris* and *Taenia* primers utilised were taken from Côté et al. (2016) but the *Macracanthorhynchus hirudinaceus* (*M. hirudinaceus*) primers were designed in this study and were based on published sequences in GenBank. MW6 was excluded from further study due to the poor shotgunning results and the lack of suitable primers in house. The targets included: *Ascaris* CytB 74bp, *Ascaris* NAD1 104bp, *Taenia* CytB 113bp, *Taenia* COX1 73bp, *Macracanthorhynchus* ND1 77bp, *Macracanthorhynchus* ND6 94bp and *Macracanthorhynchus* CytB 78bp. See Table 4.2 for further details. The *Ascaris* primers were applied to samples MW7 and MW8, the *Taenia* primers were applied to samples MW4 and MW5 and the *Macracanthorhynchus* primers were applied to MW3. The PCR product was then run on a gel to assess the results. Only the *Macracanthorhynchus* libraries were sent off for sequencing.

Before the reads were analysed, the data was cleaned. This included demultiplexing, the removal of primers, dereplication and the removal of sequences

below 20bp, which was completed using an inhouse pipeline, usearch (Edgar, 2010) and fastx (Gordon & Hannon 2010) toolkits. The sequence quality was assessed preliminarily using fastQC (Andrews, 2010). For the blast assessment, blastn (Altschul et al., 1990) was used against a compiled NCBI database and then visually assessed in MEGAN (Huson et al., 2011). Helminth hits were assessed for reliability by the number of unique reads that matched, the length of the sequence and the e-value support. The e-value represents the estimate that a hit would occur given the complexity of the query and the size of the database. On a scale of 1-0, the closer the e-value is to 0 the more robust the match is. Hits with E values above $1x10^{-10}$ were considered to be unreliable.

Finally the BWA toolkit (Li and Durbin, 2009) was applied, which uses Burrows-Wheeler transform for the rapid alignment of short fragments to reference genomes. BWA-MEM was applied using standard setting as this is the fastest and most accurate algorithm. Full mitochondria were selected for the reference genomes as these are the longest, most complete sequences available for our species of interest. In addition, there is a higher copy number of mitochondrial genomes per cell, therefore there is a higher likelihood of retrieving mitochondrial sequences in comparison to nuclear genes from degraded samples. Accession numbers of the reference sequences include: HQ704900.1 (*A. lumbricoides*), FR856886.2 (*M. hirudinaceus*), AY684274.1 (*T. saginata*), and AF216697.1 (*F. hepatica*). Upon further investigation, MW3 was also aligned against LC350001.1 (*M. hirudinaceus* 18S, ITS1, 5.8S, ITS2 and 28S genes) and manually concatenated sequences of *Macracanthorhynchus ingens* (*M. ingens*) 18S (AF001844.1), ITS1 (AF416414.1) and 28S (AY829088.1) and *M. ingens* CO1 (KT881244.1) and 16S (AF325129.3). The alignments were visually assessed using the Integrative Genomics Viewer (IGV) (Robinson et al., 2011) to visually appraise the alignment and it was quantified using the samtools flagstat command (Li, 2011).

A second novel tool has been designed that can quantify the estimate of support for a species, or genus, identification as a probability (Dimopoulos, et al. in prep). In many cases, archaeological material can be ascribed to phylum, or family, via morphological examination, but genetic analyses are required to confidently assign the taxa to species or genus. This new tool utilizes the taxonomic designation indicated by visual cues as the basis for the creation of a genetic database for comparison, by assembling already publicly available data. Every read from the archaeological material is aligned to the reference genomes and the quality match scores are stored in a matrix and subsequently get concatenated. The proportion and the alignment quality score of the sequences that can be aligned are then used to build an assignment probability. An

assignment probability over 0.5/0.6 indicates that the genetic data retrieved can be used to robustly assign a species or genus to the sample.

Due to the museum labels and the blast results we could utilise this pipeline for this study. As the assignment probability reflects the confidence that one reference genome matches to the archaeological material more closely than any of the other taxa in the database, the power of this pipeline is dependent on how appropriate and complete the reference database used for comparison is. This analysis was run multiple times with different sets of reference genomes to try and produce the most reliable results. Databases included: complete genomes for Nematodes and Platyhelminths, complete mitochondrial genomes for Nematodes and Platyhelminths, complete mitochondrial genomes for Nematodes and Platyhelminths but with the addition of a complete mitochondria for *M. hirudinaceus* and any genes available for *M. ingens* and finally just RNA genes available for *Macracanthorhynchus* species.

4.4 Results

4.4.1 Assessment of quantity and quality of DNA

The portable density meter indicated that all but one of the samples was being stored in the optimal concentration of ethanol (Table 4.1). The museum curator suggested that the collection had recently been topped up with fresh solution and therefore these concentration values were not necessarily indicative of the long-term storage environment of these samples (Mark Carnall pers comm). MW6, which was selected to explore the significance of visual indicators of genetic preservation, was the outlier as it was stored in a solution of 5.2% ethanol. Five of the seven samples had low but detectable levels of DNA from the Qubit assessment (see Table 4.3). Some studies have suggested that Qubit readings are not always the most accurate method for quantifying DNA available (Nakayama et al., 2016) so the extracts were also run through the tapestation (Agilent) to assess the concentration of sequences below 1,000bp in length. All samples, except MW3, had no detectable levels of DNA (see Supplementary 4.8.2) suggesting that the Qubit results reflected modern contaminant rather than endogenous DNA.

Sample Name	Qubit results (ng/μl)
MW1	Too low to detect
MW2	Too low to detect
MW3	0.430
MW4	0.148
MW5	Too low to detect
MW6	0.608
MW7	0.155
MW8	0.180

Table 4.3: The results of Qubit analysis on the Museum Worm extracts in ng/μl.

For the samples submitted to targeted PCR no bands were produced or primer concatemers identified in the blank, (see Figure 4.3). Despite this, reads were successfully retrieved from the Cytochrome B primer amplification of MW3, see Figure 4.4. The sequence quality retrieved from the shotgun libraries were assessed using fastQC and the results were as expected for ancient DNA**.**

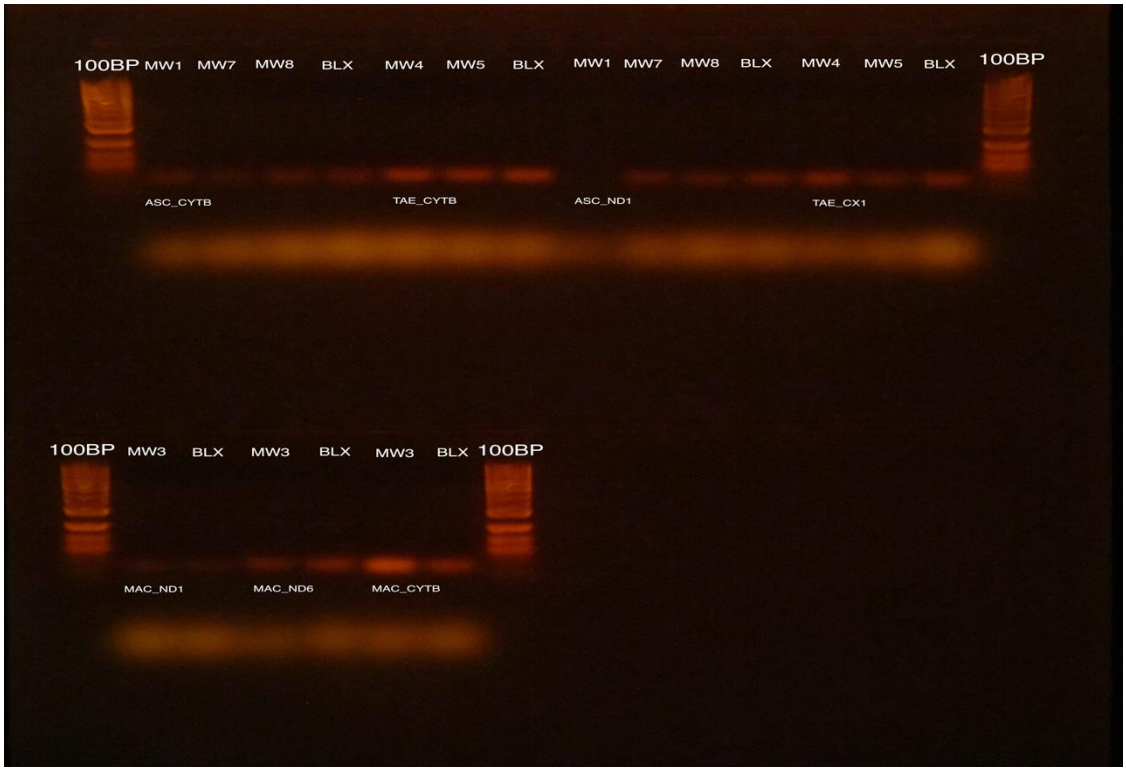


Figure 4.3: A gel run on the first round of PCR applied to the Museum Worm extracts using a number of different targeted primers and negative controls. The sample name is indicated at the top whilst the primer used is at the bottom. A 100bp ladder was used.

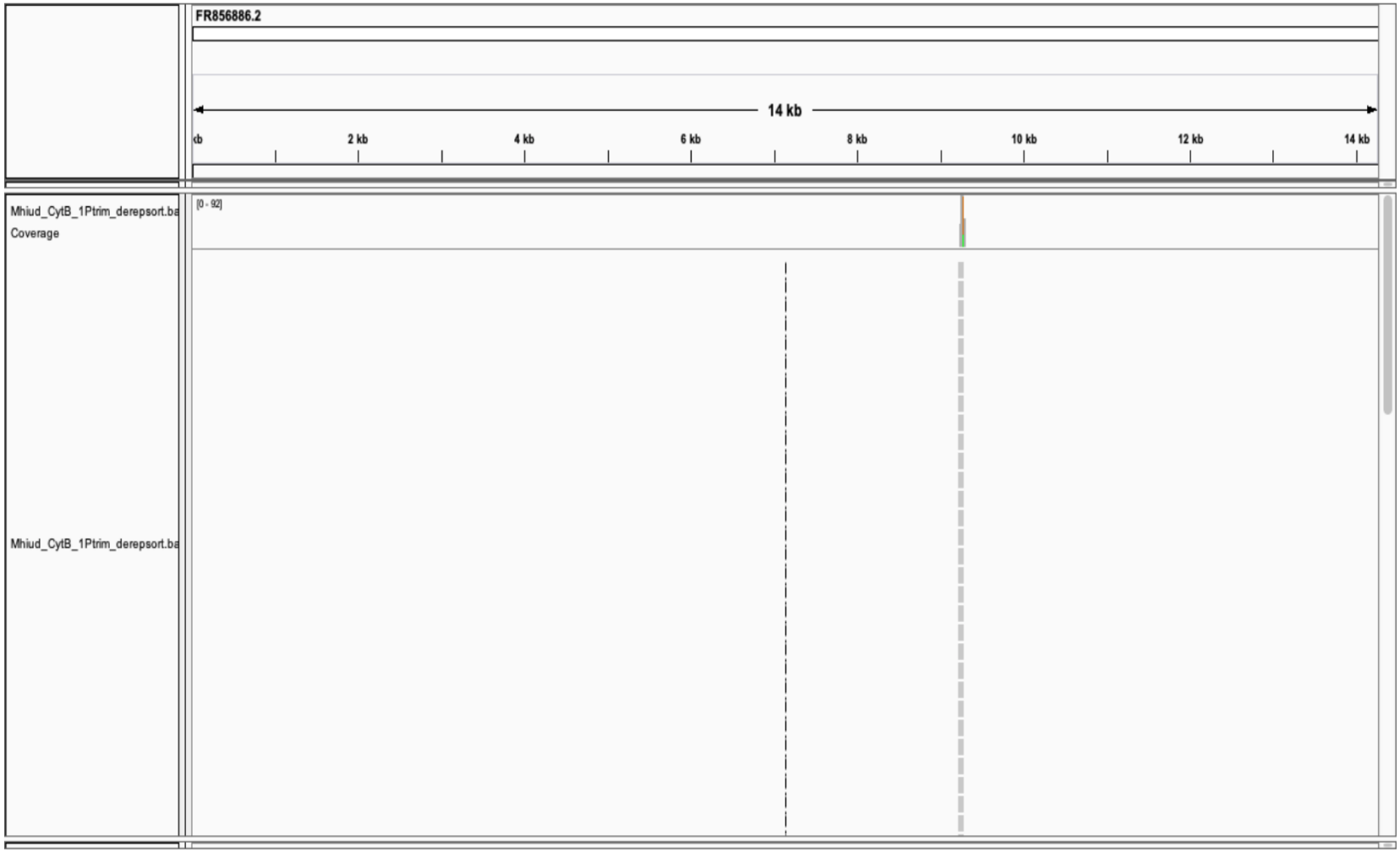


Figure 4.4: An image from the alignment of reads produced from targeted PCR analysis of MW3 using a primer that amplify a region of the Cytochrome B gene of M. hirudinaceus. This has been aligned to a M. hirudinaceus full mitochondrial genome (FR856886.2). This image was produced using IGV where grey regions indicate where fragments have aligned and coloured bars indicate bases that differ from the reference genome.

4.4.2 BlastN results

In samples MW1, MW5 and MW6 there were reads that matched to helminth reference genomes however, the number of unique sequence matches (1-2), the length of the sequences (<30bp) and the e-value support (between e-01 and e-10) were all low. In addition, some of the matches contained long strings of As at the ends which in a reference genome could indicate contaminant from library construction. There is a chance that these hits represent true matches to endogenous DNA at a very low concentration, potentially from the jar being recycled and poorly cleaned between use, but as these samples had poor quantities of DNA in the extracts the hits most likely represent sequencing by-products so were discarded from further consideration. The blast matches also identified bacteria, insects, fish and some mammals within all seven of these samples, but these appear to be either contaminant or matches based on short sequence lengths (< 30bp). See Supplementary 4.8.3 for a summary image of the hits from MEGAN.

For the other four samples there were matches that were more robust. MW7 had multiple reads matching to helminth species within the family Chromadorea however only one had an e-value support stronger than e-10, *Onchocerca flexuosa* (1 hit, 81bp, e-value= 3.318920e-33). Samples MW4 and MW8 both had reads that strongly matched to *Nector americanus* (maximum: 80bp long, e-value = 1.219300e-17 for both). MW4 had other matches within the class of Rhabditida that had weak e-value support, whilst MW8 had unique read matches that were equally as robust to *Elaeohphora elaphi* (e-value= 1.873610e-24) and *Wuchereria bancrofti* or *Gongylonema pulchrum* (e-value= 2.222150e-29) along with further matches within the family Chromadorea. Multiple polymorphisms to the modern references were detected when aligning these sequences, which could indicate that the match is to the closest taxa available in the database or there is a degree of genetic variation that is not represented in the modern reference database. The former seems likely in the case of MW8 as multiple taxa were identified by different reads. Whilst the identification of Nematode phylum fits with the museum classification of MW7 and MW8, MW4 has the morphology of a tapeworm, see Figure 4.5.

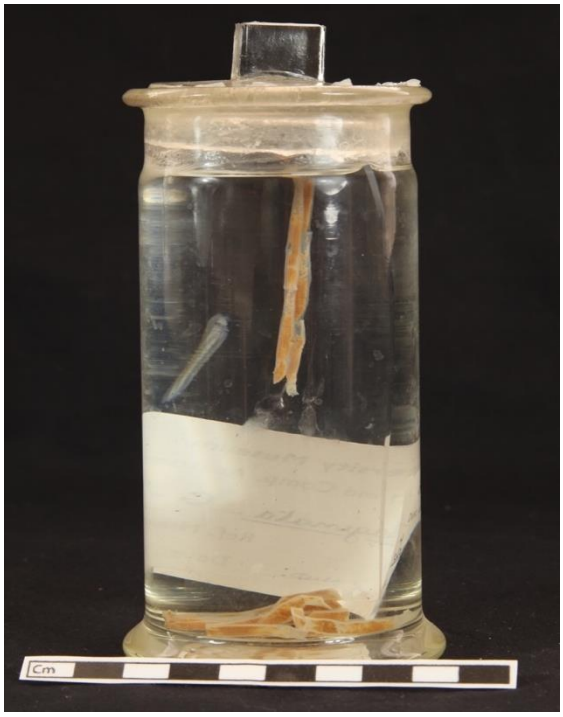


Figure 4.5: Sample MW4, identified as a tapeworm. Although damaged, it still clearly has retained the flat tape-like morphology.

Finally, for MW3 there was a clade of 16 unique reads that fell into the Archiacanthocephala class. Of these, 11 matched equally to multiple species within the class and had e-value support stronger than e-10 suggesting sequences from conserved regions of the genome had been identified. A further two hits were specifically identified as *Macracanthorhynchus ingens* with the best hits being 64bp long and having e-value of 6.177150e-24 (see Figure 4.6 for the range of blast matches). The species identification for this museum sample was *Macracanthorhynchus hirudinaceus* so the genetic results closely align to the morphological identification. For a summary of the sequences produced and the identification of endogenous DNA see Table 4.4.

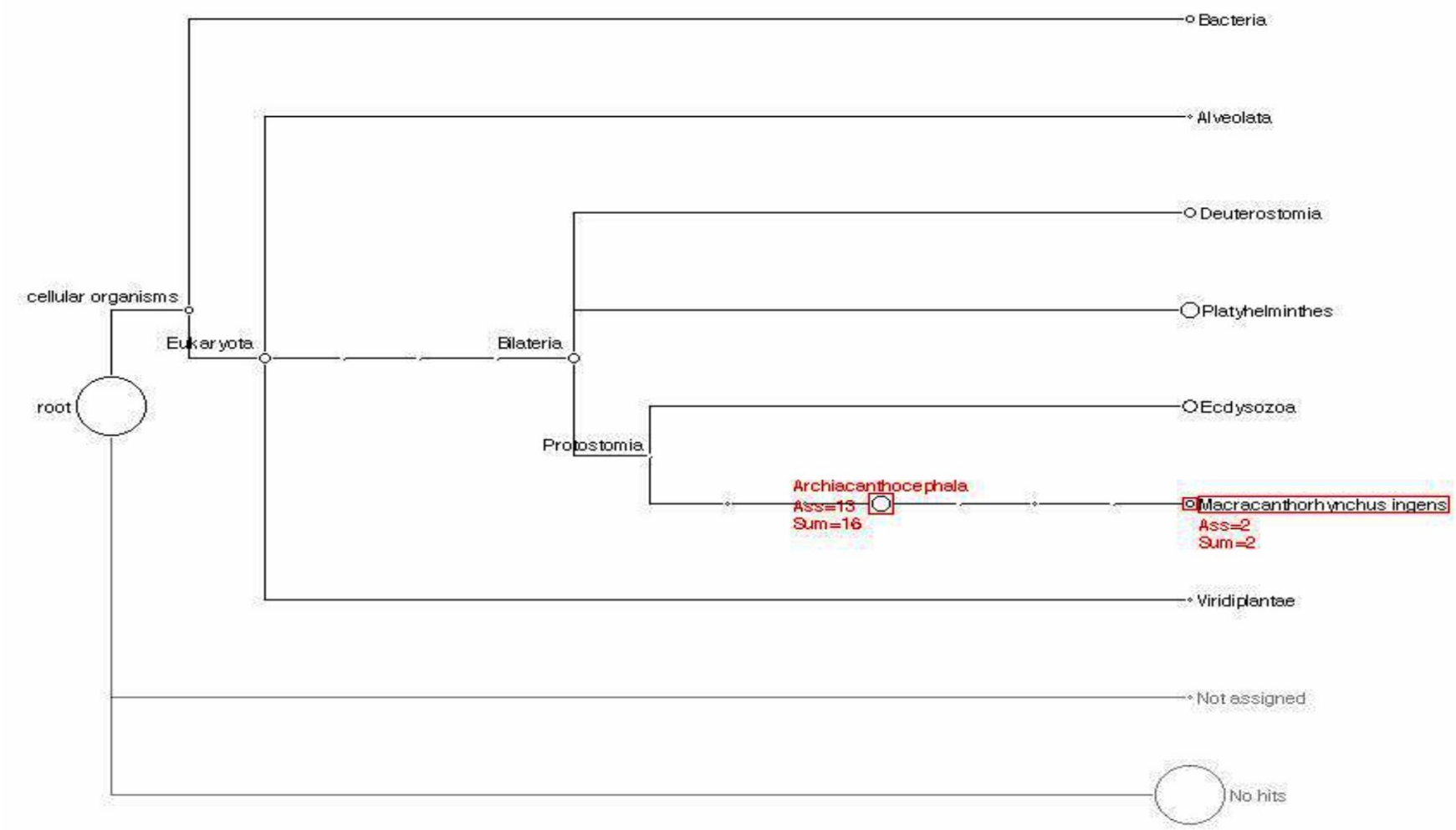


Figure 4.6: A tree displaying all the blast matches identified in MW3 in phylogenetic order. The size of the circle at the end of the branch is indicative of the number of unique hits to that class of organisms. The branches can be collapsed to show the highest groupings (such as family or order) or opened to see individual species. All branches have been collapsed except for the one which shows hits to M. ingens (2). In addition, the number of hits to Archiacanthocephala (13) has been highlighted. This is the class for M. ingens.

Sample	No. of total reads	No. of reads post-processing	Hits with E-values below e-10	All Blast hits within the same classification
MW1	188162	10174	0	0
MW3	3399918	85048	Archiacanthocephala (11 hits) *M. ingens* (2 hits)	Acanthocephala phylum (17 hits)
MW4	689925	79056	*N. americanus* (2 hits)	Rhabditida superfamily (5 hits)
MW5	660854	46164	0	0
MW6	618648	58338	0	0
MW7	727238	73569	*O. flexuosa* (1 hit)	Chromadorea class (7 hits)
MW8	830454	85554	*N. americanus* (2 hits) *E. elaphi* (1 hit) W. *bancrofti/ G. pulchrum* (1 hit)	Chromadorea class (11 hits)

Table 4.4: A summary of the shotgun results: the number of reads produced, the number of reads retained after data processing indicating the degree of clonality, the number of BLAST matches with low error rates and then all the hits that fall into the same class as the strongest matches.

4.4.3 BWA Alignment

When the shotgun reads were aligned to complete mitogenomes matching to their museum taxonomic designation, none of the samples produced successful alignments. In MW3 this directly contradicts the successful amplification of Cytochrome B sequence using the targeted approach. Due to the BLAST matches of MW3 to *M. ingens* and conserved Archiacanthocephala genes, the reads were also aligned to all published mitochondrial genes available for this species (CO1 and 16S). This also failed to produce an alignment. On closer inspection of the BLAST results, the reads from MW3 were aligned to nuclear RNA sequences from both *M. hirudinaceus* and *M. ingens* which resulted in successful alignments. Of the reference sequences available, the alignment covered 40.54% of the bases for *M. hirudinaceus* (reference length= 5,878bp: 18S, ITS1, 5.8S, ITS2, 28S genes) and 20.90% for *M. ingens* (reference length= 5,234bp: 18S, ITS1, 28S genes) when accounting for insertions and deletions (Bases Mapped CIGAR). The alignments can be viewed in Figures 4.7 and 4.8 and are summarized in Table 4.5.

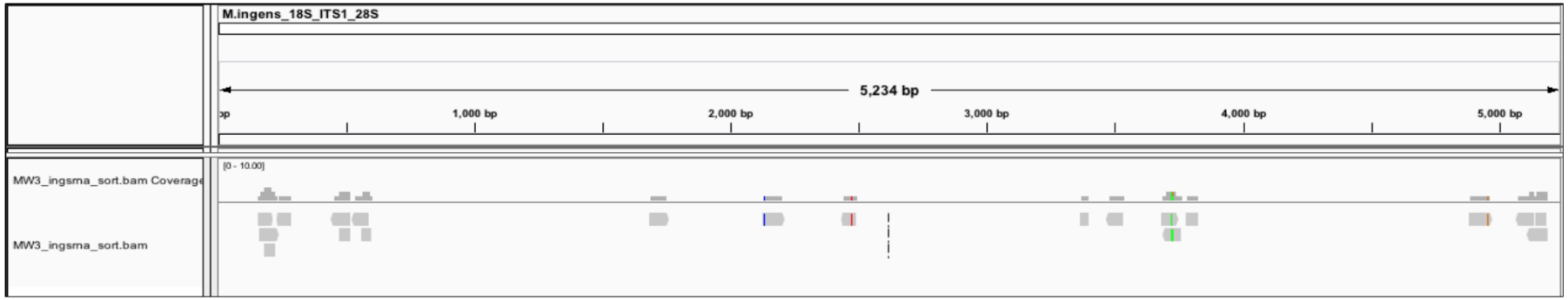


Figure 4.7: An image from the alignment of shotgun reads from sample MW3 to concatenated M. ingens 18S (AF001844.1), ITS1 (AF416414.1) and 28S (AY829088.1) reference genes as seen in IGV. Grey regions indicate where fragments have aligned and coloured bars indicate bases that differ from the reference genome.

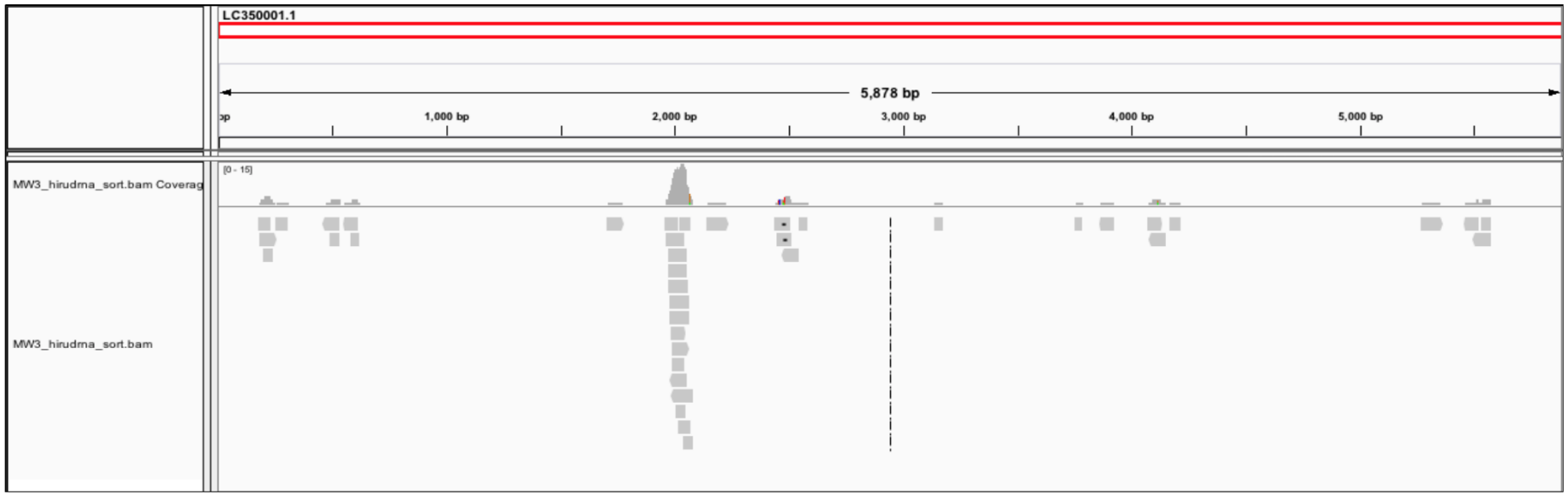


Figure 4.8: An image from the alignment of shotgun reads from sample MW3 to M. hirudinaceus 18S, ITS1, 5.8S, ITS2 and 28S genes (LC350001.1) reference genes as seen in IGV. Grey regions indicate where fragments have aligned and coloured bars indicate bases that differ from the reference genome.

Alignment species	Genes Covered	Reference length	Reads Mapped	Bases Mapped	Bases Mapped Cigar	Mismatches	Error Rate	Average Length	Maximum Length	Average Quality
M. hirudinaceus	18S, ITS1, 5.8S, ITS2, 28S	5,878	40	2383	2351	24	1.02E-02	57	81	255
M. ingens	18S, ITS1, 28S	5,234	20	1142	1094	6	5.48E-03	57	81	255

Table 4.5: Summary stats from the BWA alignment of reads, to nuclear RNA genes from both M. hirudinaceus (18S, ITS1, 5.8S, ITS2 and 28S genes (LC350001.1)) and M. ingens (18S (AF001844.1), ITS1 (AF416414.1) and 28S (AY829088.1)). Reads were produced by shotgun analysis of MW3.

4.4.4 SIGMA Pipeline

The first run utilising complete genomes suggested that 5 out of the 7 samples could be confidently assigned to *Caenorhabditis briggsae* (see Table 4.6). Considering the clear morphological differences between the museum samples, these species designations seemed unlikely. The volume and length of the reference genomes available for *Caenorhabditis* was found to be higher than in other taxa, resulting in a biased database.

Sample	1st Assignment Probability	1st Species	2nd Assignment Probability	2nd Species
MW1	0.034853701	*Caenorhabditis briggsae*	1.17E-05	*Caenorhabditis remanei*
MW3	0.981578531	*Caenorhabditis briggsae*	0.00732257	*Strongyloides ratti*
MW4	0.999946019	*Caenorhabditis briggsae*	5.40E-05	*Caenorhabditis remanei*
MW5	0.999316953	*Caenorhabditis briggsae*	0.000646377	*Pseudoterranova krabbei*
MW6	0.99999989	*Caenorhabditis briggsae*	1.09E-07	*Caenorhabditis remanei*
MW7	0.999876722	*Caenorhabditis briggsae*	0.000123278	*Caenorhabditis remanei*
MW8	0.025402617	*Caenorhabditis briggsae*	8.86E-05	*Caenorhabditis remanei*

Table 4.6: The species identified and the confidence score for the museum worms identified by the SIGMA pipeline- run 1.

The analysis was therefore re-run with a database just consisting of complete mitochondrial genomes for Platyhelminths and Nematodes to remove this bias, resulting in Table 4.7. Two samples had significant matches, MW4 and MW7. Neither of the matches corresponded to the museum species identification despite the species, and other species from the same genera, being present in the reference database. Distinct morphological differences between the museum classification and those identified using the pipeline suggested that these species matches are erroneous.

Sample	1st Assignment Probability	1st Species	2nd Assignment Probability	2nd Species
MW1	0.49334221	*Ancylostoma ceylanicum*	0.266311585	*Echinococcus ortleppi*
MW3	0.324932274	*Strongyloides ratti*	0.08311853	*Cucullanus robustus*
MW4	0.525624179	*Longidorus vineacola*	0.474375821	*Meloidogyne enterolobii*
MW5	0.452380952	*Pseudoterraova krabbei*	0.023809524	*Pseudoterranova decipiens sensu lato*
MW6	0.344530577	*Brugia malayi*	0.344530577	*Moniezia benedeni*
MW7	1	*Diphyllobothrium stemmacephalum*	None	
MW8	0.222242069	*Toxocara cati*	0.222242069	*Longidorus vineacola*

Table 4.7: The species identified and the confidence score for the museum worms identified by the SIGMA pipeline- run 2.

The next run included a complete mitochondrial genome for *M. hirudinaceus* and any reference genes available for *M. ingens* as a full mitochondrial sequence was not available. The results can be seen in Table 4.8. This produced a high degree of confidence to assigning MW3 to *M. ingens*. A number of other species were also identified as *M. ingens* but this value is based on a low number of aligning reads so it failed to produce a confident identification. As the BWA alignment identified a bias in the reads towards RNA genes, the pipeline was attempted utilising only RNA gene regions but, the pipeline failed to produce any results as an insufficient number of RNA reference sequences were available.

Sample	1st Assignment Probability	1st Species	2nd Assignment Probability	2nd Species
MW1	0.679040252	*Macracanthorhynchus ingens*	0.158342991	*Ancylostoma ceylanicum*
MW3	0.990366323	*Macracanthorhynchus ingens*	0.003130293	*Strongyloides ratti*
MW4	0.781238973	*Macracanthorhynchus ingens*	0.114986085	*Longidorus vineacola*
MW5	0.447921781	*Pseudoterranova krabbei*	0.023574831	*Anisakis berlandi*
MW6	0.33365867	*Brugia malayi*	0.33365867	*Moniezia benedeni*
MW7	1	*Diphyllobothrium stemmacephalum*	None	
MW8	0.678966569	*Macracanthorhynchus ingens*	0.071347134	*Toxocara cati*

Table 4.8: The species identified and the confidence score for the museum worms identified by the SIGMA pipeline- run 3.

4.5 Discussion

4.5.1 Quantity and quality of extracted DNA

One out of seven museum sample extracts was consistently identified as the same genus using multiple bioinformatic approaches on shotgun reads as well as by targeted PCR. This result was produced with a starting tissue weight of 93 mg and the sampling strategy meant that the specimen could still be utilised for morphological analysis in future studies. This shows that endogenous DNA can be extracted from formalin fixed museum samples over 100 years old without the complete destruction of the specimen. In samples MW4, MW7 and MW8, there were robust BLAST matches to multiple helminth taxa within the same class and family groups suggesting that endogenous helminth DNA may have been recovered, but the data retrieved could not be used for a confident genus or species identification using bioinformatic approaches. For the rest of the samples (3/7), whilst there were BLAST matches to helminth taxa the low quality of the matches results in a low confidence that endogenous DNA was extracted. It would be useful to assess a greater number of samples but due to the value of these specimens as teaching and research resources, larger sample sizes are difficult to obtain.

In our samples, there was no correlation between visual degree of degradation and endogenous DNA content. There were some specimen with seemingly excellent preservation which did not produced reliable results. This is not necessarily surprising seeing as the fixation process will preserve morphology down to the cellular level and that DNA crosslinking, denaturation and fragmentation has no visual effects. Age is likely a contributing factor to the success of an extraction however, of the two samples with associated dates in our study, the older was more successful (dating to 1886). Rather than visual cues and age, it seems likely that the dominant factor in relation to success when working with museum fluid collections such as these is storage history. Future work should therefore focus on finding museum samples with more details of their curated history and adapting the methodology based on the recorded fixation process.

4.5.2 Identification

Whilst there are multiple hits using BLAST, there is some degree of uncertainty in the reliability of these hits. This predominantly stems from the nature of the genetic database for helminths. As discussed for MW8, some of the matches identified here may be representative of the closest match of the published sequences rather than a true match. This is likely to be the case if the sequence is from a genetic locus that has only been published for some species and not others. There are further issues of uncertainty as some published sequences of parasitic worms contain contaminant either from their host species, commensal worms or from laboratory contamination (Coghlan et al., 2018). This increases the chance of false positives or misidentification of some of the

samples, which in this study may be the explanation for the BLAST identification of MW4. Despite the problems with the database, this research produces a genus identification with a strong degree of confidence for sample MW3 as *Macracanthorhynchus* due to the positive matches using BLAST, BWA and the SIGMA pipeline. Whilst the genus identification of MW3 is reliable, the incomplete nature of the published sequences and the difficulties of producing an appropriate reference database makes it difficult to ascertain the species identification.

The SIGMA pipeline has been developed specifically as a tool for species identification but this is reliant on a strong reference database. Helminth is a medical term rather than a taxonomic one, therefore multiple taxa of interest may be very distantly related. In the construction of a comparative reference database for the study of helminths, the choice is either to include a large number of closely related, but inconsequential, species that make the database large and computationally intense, or select specific taxonomic units and potentially bias the database. Due to the museum classifications, the latter option was selected.

The first run comparing complete genomes of Platyhelminths and Nematodes was heavily biased towards *Caenorhabditis briggsae* as the complete genomes are ~20 million bp whilst most other species only have complete mitochondrial genomes available (if that) and these tend to be around 15 thousand bp long. This resulted in the reference database having to be curated to target specific gene regions which is another selective bias. This became an issue for the third run. Whilst the third run confidently assigned MW3 to *M. ingens,* this is likely due to the fact that only *M. ingens* was represented by nuclear RNA genes resulting in a loci bias. This bias could also explain the positive identification of *M. ingens* for MW1 and MW4. Conversely, the database is also biased against *M. ingens* due to the short reference length of the handful of genes available in comparison to the complete mitochondrial genomes available for the other species. This makes it difficult to assess the robustness of this pipeline result.

Due to the uncertainty stemming from using different loci, the pipeline was attempted again using only RNA gene regions, but it could not run due to a lack of reference sequences, highlighting the discrepancy between the reference gene regions available for different helminths. In the *Macracanthorhynchus* species for example, there is a complete mitochondrial genome and multiple RNA genes available for *M. hirudinaceus,* but there are only a few gene regions available for *M. ingens* and no sequences available for *Macracanthorhynchus catulinus* or *Macracanthorhynchus erinacei* (taxonomy from Amin, 2013). There is a general lack of published helminth

whole genomes due to their large and complex nature (Wit and Gilleard, 2017). Whilst this is a limitation, the decreasing costs of whole genome sequencing and the rise of third generation sequencing may mean that the approach developed here may be more successful in the future.

4.5.3 Phylogeography

With the data produced here and the incomplete nature of the genetic database for helminths, it seems unlikely that a wide scale analysis of museum worms could currently be utilised for phylogeography. The success rate in this study for species identification (1/7) indicates that a large number of museum samples would need to be damaged to produce a functional reference database. The goal of future research should be to improve the proportion of endogenous reads recovered, then this ratio of successful samples would be more justifiable.

4.6 Conclusion

This study has demonstrated that there is endogenous DNA that can be sequenced within formalin fixed museum samples that are over 100 years old. The data retrieved can be utilized to assess whether the taxonomic identification in the museum is accurate which is a useful tool for curators working with unidentified fluid stored samples. Despite this success, the quality and quantity of the sequences we retrieve back, in addition to the incomplete nature of the reference databases, makes species identification difficult and phylogeographic analysis unlikely for now. To make the utilisation of museum samples to build a near recent reference database viable, positive samples would need to produce a greater number of on-target hits. One option for addressing this is to use larger tissue samples, however this approach may reduce the likelihood of curators agreeing to sampling.

Future research should instead explore genetic enrichment for fluid collections, as this approach has the capacity to increase the number of endogenous reads without a change to the sampling strategy. A hybrid capture-based enrichment approach has multiple advantages over shotgunning such as: a reduction in allelic loss, targets can be designed to avoid paralogs, complexity, and repetitive regions and it allows for multiplexing of multiple genera (da Fonseca et al., 2016; Heyduk et al., 2016). The disadvantage is that there is variation in enrichment efficiency, somewhat dependent on probe design and sample quality (Hosner et al., 2016). The ability to design successful probes is reliant on the complexity of the genomes of interest and the quality and quantity of the published sequences (Heyduk et al., 2016).

Currently, the most informative and complete published sequences for helminths are the mitogenomes which have the capacity for both species identification and making inferences on phylogeography. With further publication of complete reference genomes, the capacity of baiting would be even greater. In addition to this, bioinformatic results would be more robust due to the greater number of references available for comparison. With the rise of third generation sequencing platforms for de novo assembly of complex genomes (Eccles et al., 2018), it is likely that the number of published helminth genomes will drastically rise in the near future. These benefits would make working with museum samples for the development of helminth phylogeography more fruitful. It seems likely therefore that these organisms could soon be utilised to understand the spread of disease and as a proxy for understanding human migration.

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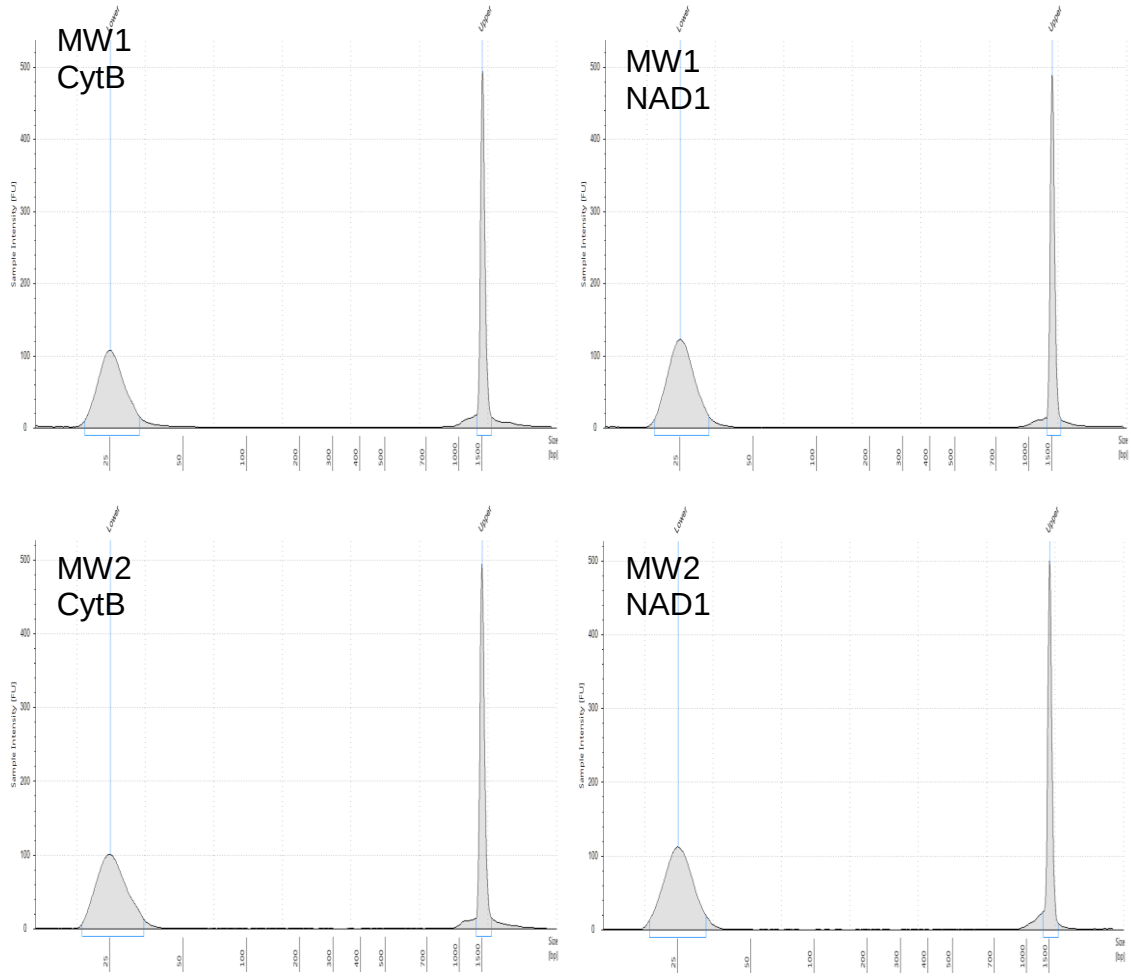
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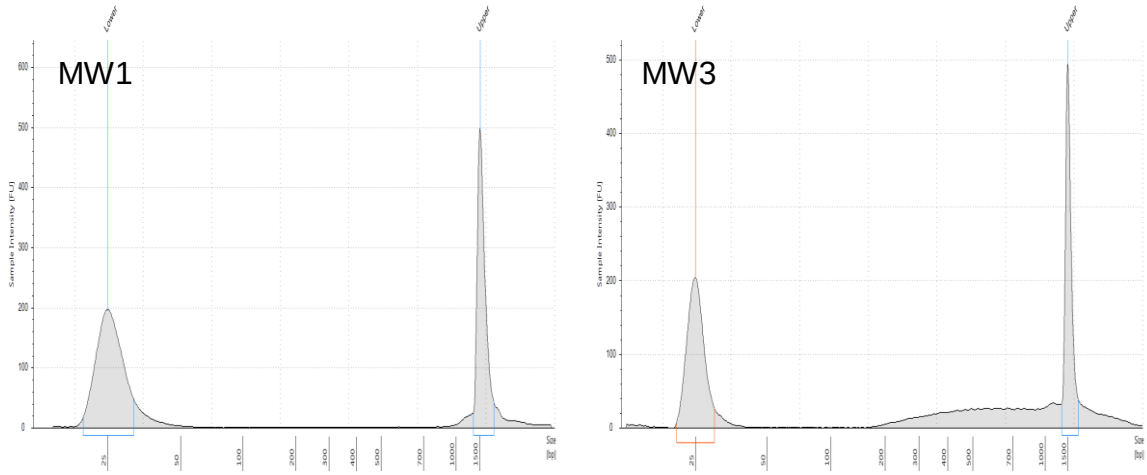
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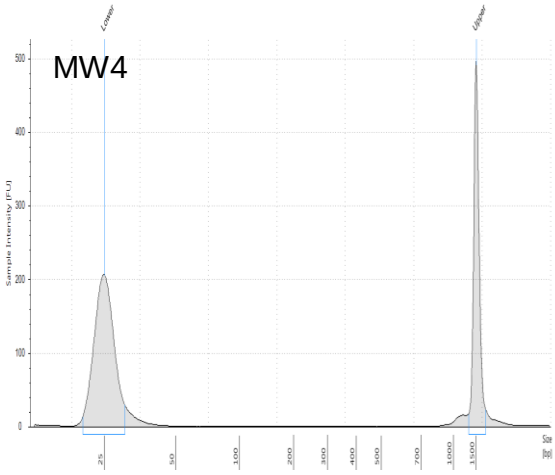
4.8 Supplementary

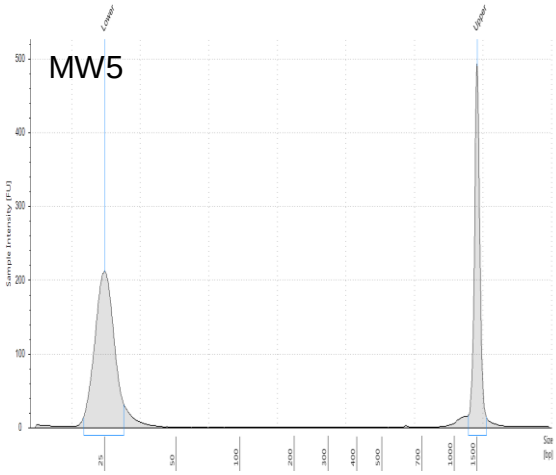
4.8.1 4 images of the tape station electropherogram of MW1 and MW2 using targeted PCR using *Ascaris*-specific primers targeting CytB and NAD1 genes.

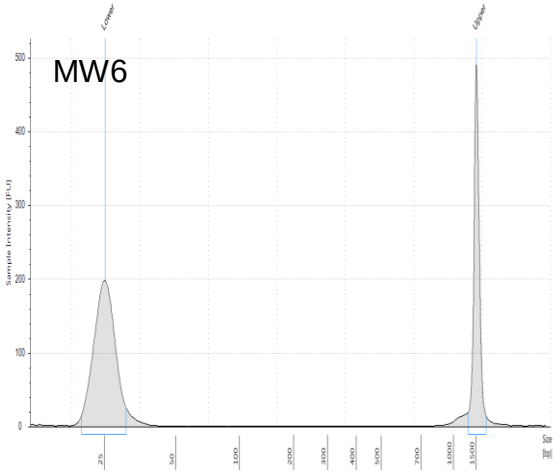


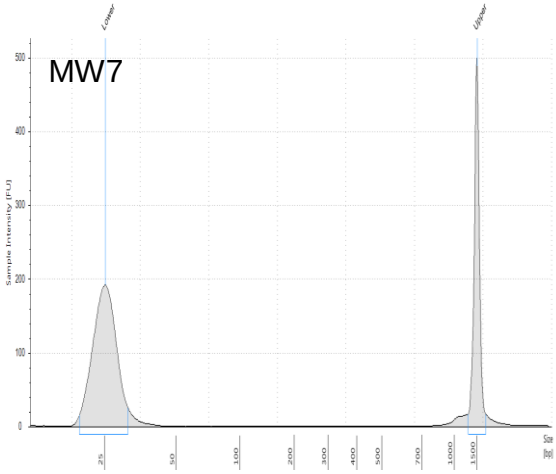
4.8.2 Images of the tape station electropherogram of MW1- MW8 extracts. All samples except MW3 had no detectable levels of DNA.

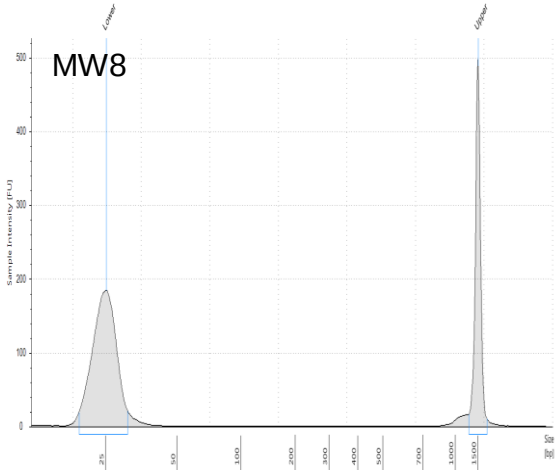



MW4
Lower
Upper
Sample Intensity (FU)
25
50
100
200
300
400
500
700
1000
1500

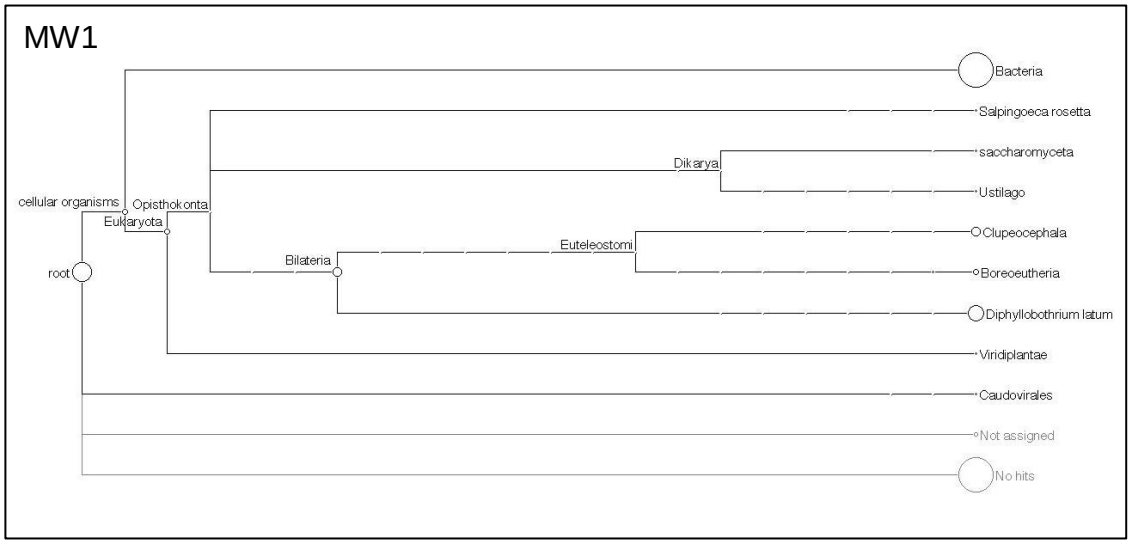

MW5
Lower
Upper
Sample Intensity (FU)
25
50
100
200
300
400
500
700
1000
1500

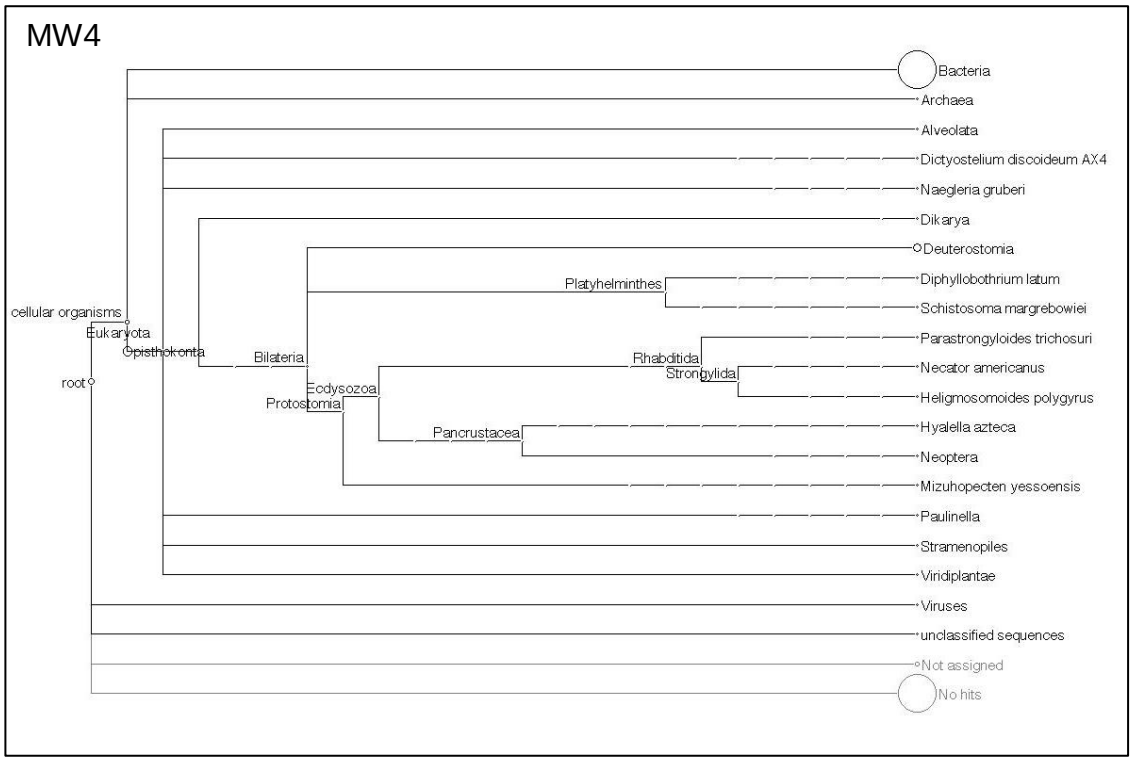

MW6
Lower
Upper
Sample Intensity (FU)
25
50
100
200
300
400
500
700
1000
1500


MW7
Lower
Upper
Sample Intensity (FU)
25
50
100
200
300
400
500
700
1000
1500

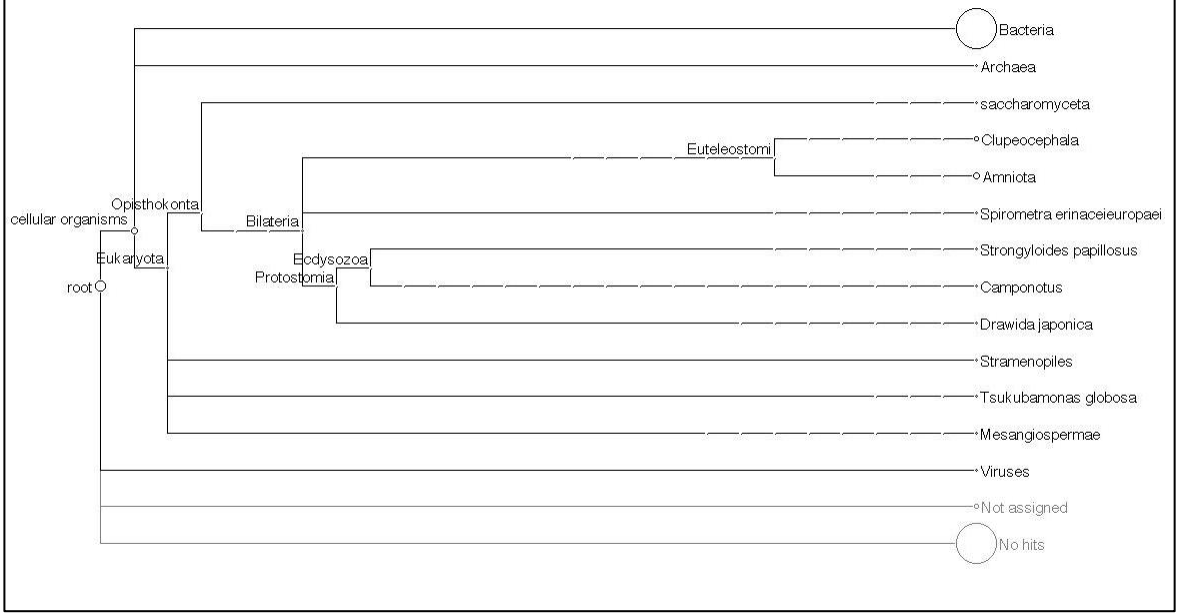

MW8
Lower
Upper
Sample Intensity (FU)
25
50
100
200
300
400
500
700
1000
1500

4.8.3 Image of the hits from MEGAN for every extract MW1-8 (excluding MW3 which is Figure 4.6). The tree has been collapsed to make the identification of helminth reads clearer.

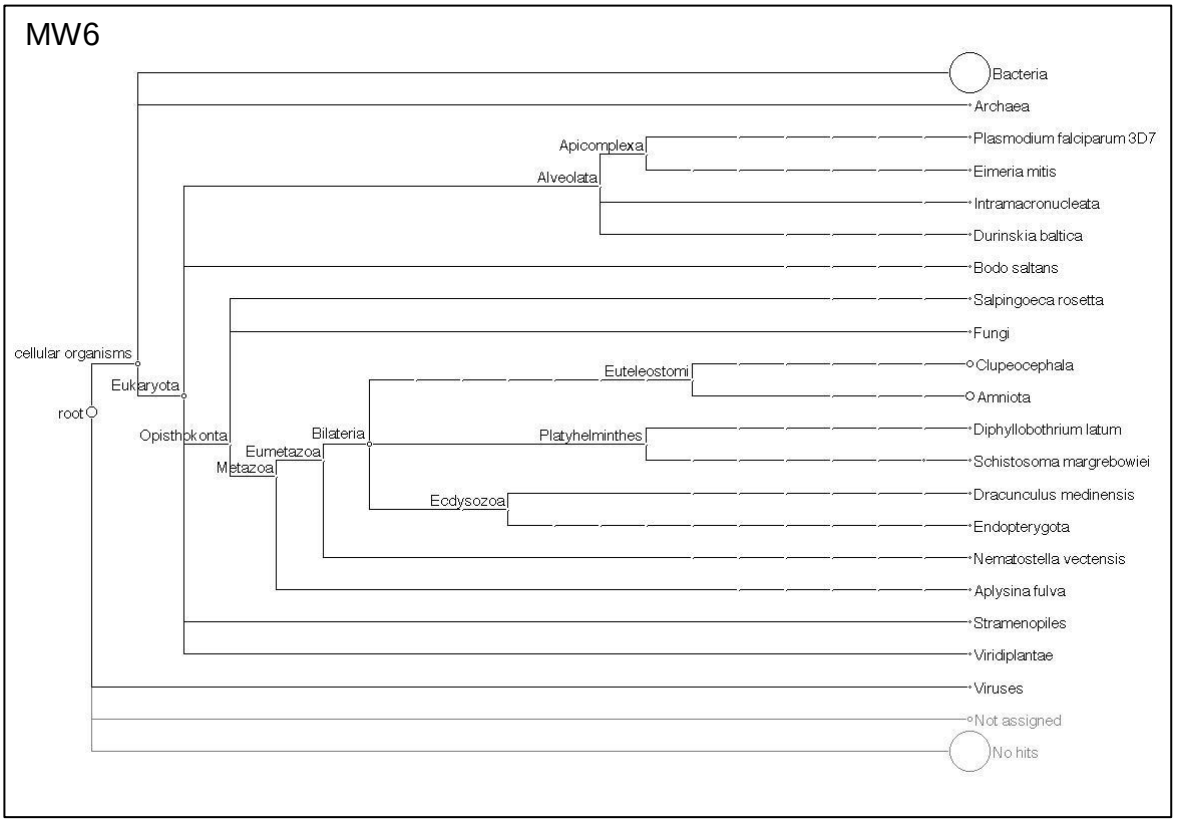




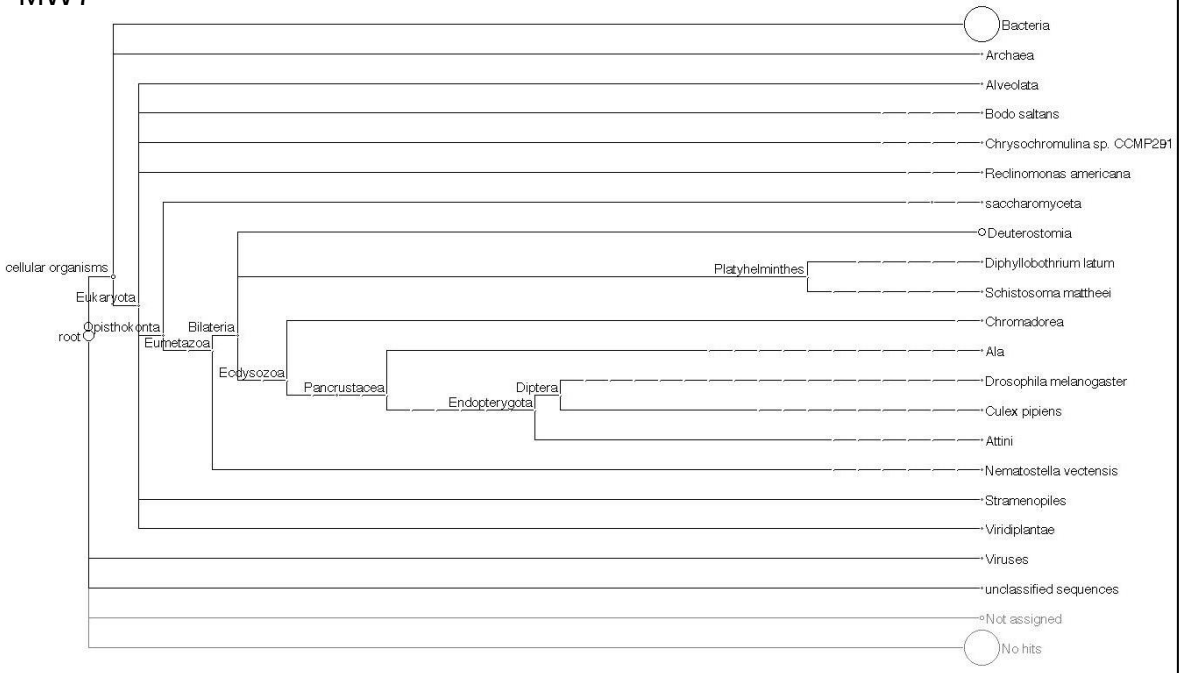
MW5


Bacteria
Archaea
saccharomyceta
Euteleostomi
Clupeocephala
Amniota
Opisthokonta
cellular organisms
Bilateria
Spirometra erinaceieuropaei
Eukaryota
Ecdysozoa
Strongyloides papillosus
Protostomia
root
Camponotus
Drawida japonica
Stramenopiles
Tsukubamonas globosa
Mesangiospermae
Viruses
Not assigned
No hits

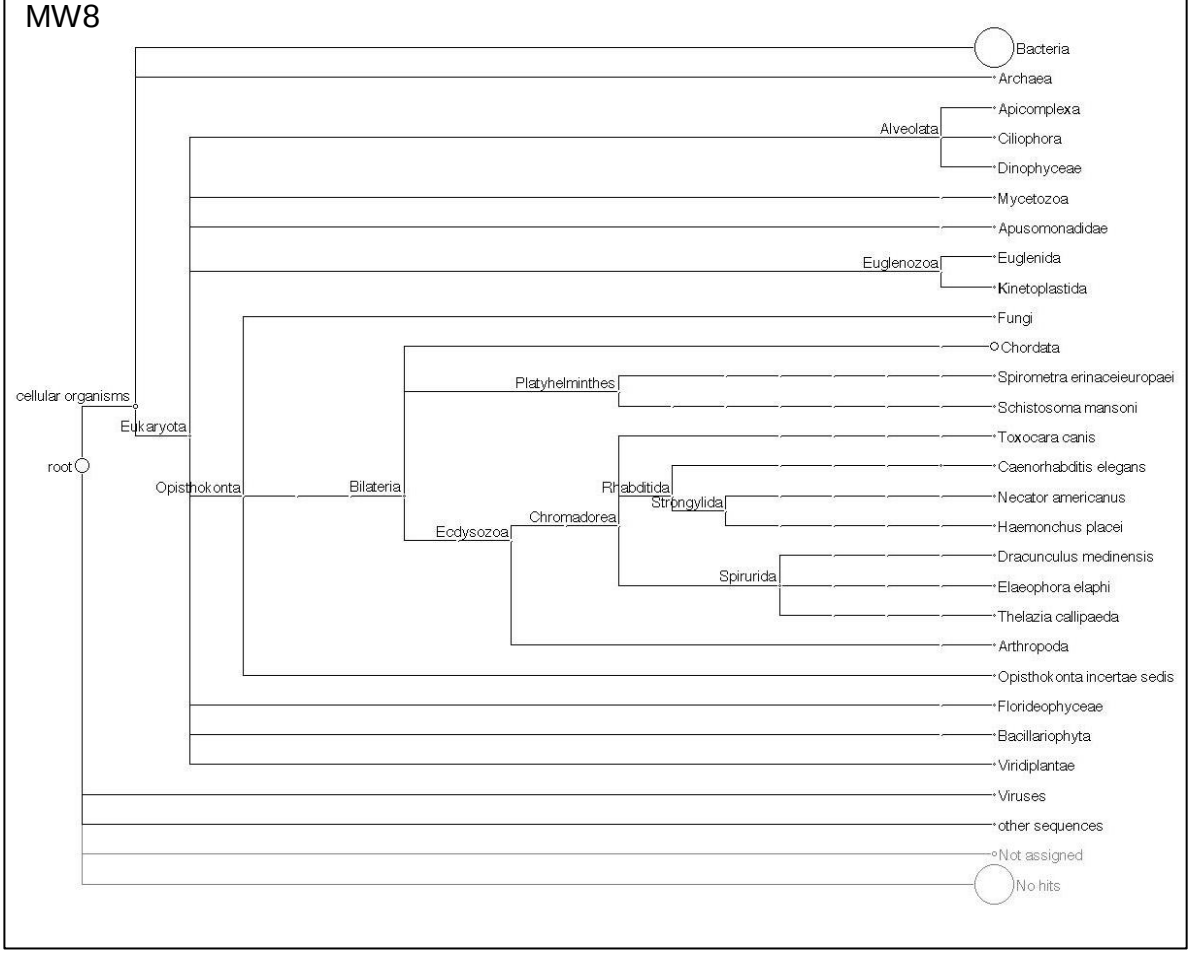
MW6


Bacteria
Archaea
Apicomplexa
Plasmodium falciparum 3D7
Alveolata
Eimeria mitis
Intramacronucleata
Durinskia baltica
Bodo saltans
Salpingoeca rosetta
Fungi
cellular organisms
Euteleostomi
Clupeocephala
Eukaryota
Amniota
root
Opisthokonta
Bilateria
Platyhelminthes
Diphyllobothrium latum
Eumetazoa
Schistosoma margrebowiei
Metazoa
Ecdysozoa
Dracunculus medinensis
Endopterygota
Nematostella vectensis
Aplysina fulva
Stramenopiles
Viridiplantae
Viruses
Not assigned
No hits

MW7


Bacteria
Archaea
Alveolata
Bodo saltans
Chrysochromulina sp. CCMP291
Reclinomonas americana
saccharomyceta
Deuterostomia
Platyhelminthes
Diphyllobothrium latum
Schistosoma mattheei
cellular organisms
Eukaryota
Opisthokonta
root
Bilateria
Eumetazoa
Chromadorea
Ala
Ecdysozoa
Pancrustacea
Diptera
Drosophila melanogaster
Endopterygota
Culex pipiens
Attini
Nematostella vectensis
Stramenopiles
Viridiplantae
Viruses
unclassified sequences
Not assigned
No hits

MW8

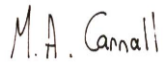

Bacteria
Archaea
Apicomplexa
Alveolata
Ciliophora
Dinophyceae
Mycetozoa
Apusomonadidae
Euglenozoa
Euglenida
Kinetoplastida
Fungi
Chordata
Platyhelminthes
Spirometra erinaceieuropaei
Schistosoma mansoni
cellular organisms
Eukaryota
Toxocara canis
root
Caenorhabditis elegans
Opisthokonta
Bilateria
Rhabditida
Necator americanus
Strongylida
Chromadorea
Haemonchus placei
Ecdysozoa
Dracunculus medinensis
Spirurida
Elaeophora elaphi
Thelazia callipaeda
Arthropoda
Opisthokonta incertae sedis
Florideophyceae
Bacillariophyta
Viridiplantae
Viruses
other sequences
Not assigned
No hits

4.9 Permission from co-authors.
I hereby give permission to Hannah F Ryan to use our joint work as contribution towards her D.Phil. thesis to be submitted for examination at Oxford University.

I confirm that to the best of my knowledge, the author contribution statement below is accurate and Hannah F Ryan's contribution towards the work is greater or equal than that of any other co- author.

Chapter author contribution statement: H.R., P.F., A.S. and G.L. designed the research; H.R. collected samples with aid from A.S.; M.C. provided samples with museum contextual information; H.R. developed the genetic extraction protocol with input from P.F.; H.R. carried out the genetic extractions and quality tests; P.F. and H.R designed primers with input from A.S.; H.R. ran targeted PCR and gel electrophoresis; H.R. carried out the HiSeq library build; P.F. carried out the MiSeq library build; L.F. and S.P developed and carried out demultiplexing pipelines, H.R. carried out the data processing and alignment of reads using BWA, H.R. carried out shotgun analysis, E.A.D. developed and implemented the SIGMA pipeline with input from L.F., A.S. and G.L. secured funding for the project; H.R. wrote the initial draft of the manuscript with input from E.A.D., P.F., A.S. and G.L.

Date: 29/3/2019
Name: Mark Carnall

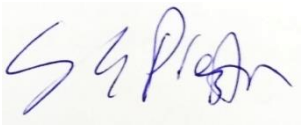


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Chapter Five: Development of Genetic Approaches to Palaeoparasitology for tracing population dynamics

5.1 Abstract

Genetic approaches to palaeoparasitology is an emerging field. Currently it is predominantly used to confirm the results of microscopy, however there is the potential to use genetics to understand population dynamics and dispersal of these species. A question remains as to whether the fluctuations in prevalence rates of helminths in the UK over time, found in Chapter 3, were site specific or a reflection of the broader population. To resolve this, a genetic approach that was sensitive to the detection of *Ascaris* spp. and capable of reliably detecting variable genetic regions that could be used to infer dispersal or population dynamics would need to be established. Our study found that whilst shotgunning can be used to identify multiple helminth species within an archaeological context, a targeted PCR approach was more sensitive in the detection of *Ascaris* spp. positive samples and would therefore be more appropriate for a widescale study. Published primers for this taxon were found to be unsuitable for questions on past variation so new primers were developed however, these were not successful. This study highlights both the strengths and weaknesses of the shotgunning and targeted PCR approaches to palaeoparasitology which can be used to direct future researchers towards key areas for development.

5.2 Introduction

Microscopy has been the main technique utilised for palaeoparasitology for as long as it has been recognised as a discipline. It has been used for assessing the pervasiveness of helminths throughout history so we can understand health (Fisher et al., 2007), hygiene (Mitchell, 2016), subsistence (Knights et al., 1983) and in a small number of cases, migration (Araújo et al., 1988; Le Bailly and Bouchet, 2010). This methodology can be used for latrines, coprolite and skeletal pelvic sediment analysis and is still the most accurate method for quantifying eggs present at an archaeological site. Microscopic identification however, is usually limited by the preservation of the eggs, the soil type, the skill of the researcher and, in many cases can only reliably be used to detect the genus of the helminth, not the species (Dittmar, 2009).

In our study of parasitology in the UK (Chapter 3), microscopy was used to detect fluctuations in the prevalence of helminths in the population over time. These fluctuations suggest that the significance of helminths as a health problem in historical populations was impacted by changes to the accumulation of waste and the practices and infrastructure designed to manage it. This fluctuating pattern was predominantly

influenced by *Ascaris* spp. due to the highest recovery of these eggs. This study is the first to build a temporal narrative of parasitology for a country however, uncertainty remains as to whether the pattern detected is site specific or representative of broader changes over time. Genetic analysis has the potential to provide complimentary data to identify helminth population dynamics (Côté and Le Bailly, 2017) which could resolve the outstanding questions from our earlier study.

Considering the number of ancient genetic studies published on humans, animals and even bacteria and viruses, the number of studies utilising this technique on helminths remains low. Of this small number, the majority utilise targeted PCR to confirm microscopic identification and potentially identify the eggs down to the species level. The study by Flammer et al. (2018) differs through the design of primers that targeted a variable ITS1 region in *Trichuris trichiura* (*T. trichiura*) which was then successfully used to identify connectivity between two Medieval trading ports. Whilst the targeted approach is the best established, it does not necessarily mean it is optimal for palaeoparasitological studies. There are two further published genetic approaches that have been developed and used for palaeoparasitology: multiplexing PCR (Côté et al., 2016) and a combination of floatation and shotgun sequencing (Søe et al., 2018).

Multiplexing is a high throughput approach but, the protocol in Côté et al. (2016) was designed for the recovery of multiple species. Whilst the authors report uncovering novel genetic variants, as the primers were designed to be shorter than 80bp and have the capacity to discriminate between 16 different species of gastrointestinal parasites, they have not been optimised for tracing population dynamics. The method used in Søe et al. (2018) on the other hand was successful for the reconstruction of complete mitochondria of *Ascaris* spp. but their protocol required high starting soil weights, utilised floatation and the consensus mitochondria were built using mixed communal samples. High soil weight combined with the likely loss of eggs during floatation (Reinhard et al., 1987, 1986) makes this protocol unsuitable for single grave analysis, or any fine scaled assessment that would require the use of small soil samples. Building consensus mitochondria is also problematic in that it minimises the genetic variation recovered, as only the most common variants will be retained. With a different extraction technique than the one outlined by Søe et al. (2018) however, shotgunning has the potential to be better at the recovery of helminth DNA from sedimentary contexts than the targeted approach.

Shotgunning is a non-selective approach that is not reliant on published sequences for its design, whereas amplicon sequencing produces high amounts of

genetic material and with good primer design, a low number of the reads will be environmental contaminant DNA (Rizzi et al., 2012). The assessment of the 'best' of these two approaches for the analysis of genetic diversity of helminths over time falls into two categories. Firstly, which of the approaches is the best for the positive identification of *Ascaris* spp. DNA from archaeological samples, particularly single graves, and secondly can either of these approaches recover genetic sequences with a high degree of variation that can be used for modelling population dynamics. *Ascaris* spp. is the main focus for this study as it was most commonly recovered from English single graves, see Chapter 3.

To establish the best tool for identification, a preliminary test on the capacity for shotgunning sequencing to recover any helminth DNA without the pre-processing steps used by Søe et al. (2018) was undertaken (Test 1). This was used to inform the best practice for the following direct comparison of the targeted approach against shotgunning using the same extracts (Test 2). The approach that was strongest for the identification of *Ascaris spp.* DNA was then developed to improve the recovery of variable genetic regions (Test 3). This final test was applied to soil samples which had previously been confirmed to have *Ascaris* spp. both by microscopy and PCR as a final test to confirm the capacity and reliability of the approach. Not only will this study highlight the strength and weaknesses of using these two sequencing approaches for palaeoparasitology, but it will also establish the feasibility of a wide scale study to establish *Ascaris* population dynamics over time. These steps will move the study of helminths to a level that begins to be comparable to research on other pathogenic organisms in the archaeological record.

5.3 Overview of Methods used to compare Targeted PCR and Shotgunning Sequencing (Test 1 and 2)

5.3.1 Extraction Methodology

5.3.1.1 Extraction over view

For Test 1, two sediment extraction protocols were used to compare which was better suited for palaeoparasitological analysis. The first is the sediment protocol described by Flammer et al. (2018) and the second is the protocol described by Haile (2012). In addition, 12 of the samples (TNB9621, TB12534, ES14.1, RHAF23264, PREDW1, COLL28353-6, COLL28350-5, CAM2014A62860, COVA2978, ES14.2, RAQ10H18, TEM1) used in this test arrived in water so an extraction protocol based on the buffers designed by Dabney (2013) was applied to identify any soluble DNA in the solutions. Details of the reagents used can be seen in Supplementary 5.10.1.

Due to the lack of success with the Haile (2012) protocol, and as all other samples arrived dry and were unsuitable for the Dabney (2013) protocol, the Flammer et al. (2018) protocol was utilised for the other two tests. For Test 2 however, four variants of the Flammer (2018) protocol was applied to see whether the protocol could be improved upon. The first variation was to either use the established Qiagen DNeasy Blood and Tissue extraction kit or the DNeasy Mericon Food kit. Whilst these protocols are relatively similar, the Qiagen DNeasy mericon Food kit has a chloroform separation step. In addition to using these 2 kits, we also repeated these extractions but replaced the kit columns (designed for modern samples) with Qiagen MinElute (designed for the retention of short sequences). Whilst this is only a minor change, this might make a noticeable difference to the ratio of ancient (short) to modern sequences (long) and improve the recovery of ancient reads. To summarise, the four variants are: Blood and Tissue kit using the kit column, Blood and Tissue kit using a Qiagen MinElute column, Food kit using the kit column and Food kit using a Qiagen MinElute column.

5.3.1.2 Haile (2012) Sediment extraction protocol

Around 2.4 ml of bulat buffer was added to 5-7 gm of unfiltered sediment and mixed. 1.5 ml of this solution was added to tubes which were quarter filled with glass beads. The eggs were then broken down in a bead beater in a run lasting 5 minutes before the samples were incubated for an hour at 65 °C. Samples were then centrifuged for 5 minutes before 300 µl of phenol-chloroform were added. Thorough vortexing was applied to the samples before they were left to stand at room temperature for 10 minutes. After centrifuging, the aqueous phase was removed and mixed with 22.5 ml of PB buffer. This was then run through a minElute spin column through centrifuging. 500 µl of Salton Buffer was then run through the spin column for 1 minute, followed by two runs of 500 µl of wash buffer. After a dry run, 40 µl of AE elution buffer was added to the spin column and left for 2 minutes to percolate before being run through a centrifuge for 1 minute. This step was repeated resulting in 80 µl of extract.

5.3.1.3 Flammer et al. (2018) Sediment extraction protocol

5-7 gm of soil were weighed and mixed with 20 ml of MilliQ-water. The sample was then vortexed and left-over night to disaggregate. The sample was then centrifuged for 5 mins at 400 G before floating material was poured off. The pellet was then re-suspended in 20 ml MilliQ-water. Large material was excluded through multiple sieving steps. Nylon sieves of aperture size 1030, 500, and 100 µm were sequentially used to avoid clogging and to retain parasite eggs that range between 20-70 µm. Samples were then centrifuged as before being re-suspended in 2 ml Tris-HCl buffer (pH 7.5). This solution was then added to tubes which were quarter filled with glass beads. The eggs

were then broken down in a bead beater in runs lasting 5 minutes, before a standard column-based extraction protocol was used on the supernatant. Unless otherwise stated, the Qiagen DNeasy Blood & Tissue kit was used.

5.3.1.4 Water extraction protocol based on the protocol outlined in Dabney (2013)

For each water sample, approximately 10 ml of Binding Buffer and 400µl 3M sodium acetate was added and mixed by gentle shaking. The volume capacity of MinElute tubes was amplified by inserting an extension reservoir of a Zymo V-spin column into the opening and placing this assembly into a 50 ml falcon tube. The sample/binding buffer mixture was poured into the extension reservoir before being centrifuges for 4 minutes at 1,500 rpm. The Falcon tubes were then rotated before being centrifuged again for a further 2 minutes at 1,500 rpm. The MinElutes were then removed from the Zymo extension and placed back into the collection tube for a dry spin for 1 min at 6,000 rpm in a table-top centrifuge. 700 µl of PE buffer was then added before the samples were spun for 30 seconds at 6000 rpm. This step was repeated. Another dry spin was run, this time for 1 minute at maximum speed. 42 µl of TET was then added to the top of the silica membrane and left to stand for 2 minutes. This was then spun for 30 second at maximum speed. Again, the step was repeated to produce an extract of ~ 80 µl.

5.3.2 Sequencing approaches

Shotgun sequencing was applied in Test 1 and Test 2, whilst amplicon sequencing was applied for Test 2. Details of the reagents used can be seen in Supplementary 5.10.1.

5.3.2.1 Targeted PCR

The targeted PCR steps were based on those outlined by Flammer et al. (2018). Two rounds of PCR were undertaken: the first round used template DNA whilst the second round used 5 µl of the first round PCR solution as the template. The library build consisted of 40 cycles per round, with the Tm of the primers dictating the annealing temperature. In Test 2, the primers used had been previously published including *Ascaris lumbricoides* CO1 (241 bp), *T. trichiura* ITS1 (459 bp), *Taenia solium* COX1 (202 bp), *Taenia saginata* COX1 (199 bp) and a universal eukaryote mitochondrial 16S (Horreo et al., 2013) and Cryptosporidium 18S primer as two non-specific targets to detect PCR inhibition (all primer details can be seen in Supplementary 5.10.2). Gel electrophoresis was used as a preliminary assessment of the success of the amplification. Samples were then selected, pooled and then cleaned using a column

(MinElute, Qiagen). They were then prepped for sequencing using the NEB- Next Ultra DNA Library Prep Kit for Illumina. The libraries were sequenced on a MiSeq.

5.3.2.2 Shotgun approach

Adaptor Ligation was carried out based on the methodology in Meyer and Kircher (2010). After the adaptor ligation, QPCR was undertaken to roughly quantify the amount of endogenous DNA that was present in the samples. The samples were then run through Thermo-Fisher StepOne Real-Time PCR System for QPCR and the results were used to assess which samples should be further processed and the optimum number of cycles for the PCR amplification. Once the library was built, an Agilent Tape station was used to quantify the distribution and concentration of different fragment lengths. An assumption that endogenous ancient DNA will have fragment lengths between 150-300bp long was used when pooling samples. During the pooling, the samples were cleaned again using Qiagen silica columns. Pooled DNA was then run through the Sage Pippin Prep machine which was used to isolate DNA fragments between 150-300bp long. The retained DNA was then run again through the Tape Station to confirm the average length and concentration of the DNA before it was pooled and sent off for sequencing on an Illumina HiSeq at Copenhagen.

5.3.3 Data processing

For reads received from the MiSeq, they were demultiplexed and initially processed using a pipeline developed by Stephen Preston. For reads received from the HiSeq, they were demultiplexed and initially processed using a pipeline developed by Laurent Frantz. To process the reads, multiple toolkits and software were used including: Trimmomatic (Bolger et al., 2014) for removing illumina primers and removing sequences below 36 bp for MiSeq runs, 25 bp for HiSeq, Usearch (Edgar, 2010) for dereplication, UrQT (Modolo and Lerat, 2015) for the removal of PolyA tails, FastQC (Andrews, 2010) as a preliminary assessment of the quality of the HiSeq runs, Burrows-Wheeler Aligner (Li and Durbin, 2009) and the Interactive Genome Viewer (Robinson et al., 2011) for the assessment of the targeted PCR results and, Blastn (Altschul et al., 1990) and MEGAN (Huson et al., 2011) for the assessment of the shotgun sequencing. The database used for BLAST was a combination of the NCBI database and sequences available from wormbase (https://parasite.wormbase.org). This database was downloaded and compiled March 2016. The commands used can be seen in Supplementary 5.10.1.

5.4 Overview of Material used to compare Targeted PCR and Shotgunning Sequencing (Test 1 and 2)

Due to the number of tests carried out in this study, a wide range of samples were used, see Table 5.1. All 31 samples had undergone microscopic analysis and 10 of them had also successfully produced *Ascaris* spp. and/or *T. trichiura* reads using targeted PCR prior to the start of this study. These samples predominantly fell into two classes, communal soil samples (n=15) and single burial soil samples (n=13). The former is useful for testing different methods as they contain a high density of eggs and therefore they are more likely to be successful for the amplification of endogenous genetic material. Single burial samples are more difficult to work with; however the key advantage of these contexts is that there is a direct association between the data retrieved and the human host. The samples analysed in Chapter 3 are single burial samples so the methods developed here have to be sensitive enough to resolve questions of population dynamics for these types of sample. Coprolites are a third sample type which is used for palaeoparasitology however, these are recovered at a much lower rate than sediment samples. Three samples in this study were coprolites but the sample sizes were too low to use them in multiple tests.

There were two tests to establish whether shotgunning or targeted PCR was a stronger approach for palaeoparasitology: Test 1- Preliminary Shotgunning Test and, Test 2- Direct comparison of shotgunning and targeted PCR. For both tests, both of the common contexts (communal and burial sediment) were utilised however, depending on the overall goal the proportion of these contexts varied. The goal of Test 1 was to establish the capacity of shotgunning for the identification of helminth DNA in soil samples so the majority of the samples utilised were communal to maximise the number of positive results. Single grave samples were still included in these tests however, as an initial indicator of the sensitivity of these methods. Test 2 on the other hand was designed specifically to compare shotgunning and targeted PCR for single burial analysis and therefore all but one of the samples came from a burial context (one communal sample was included as a positive control). The three coprolite samples were used in Test 1. As this was the most exploratory of the three tests, the density of the eggs in the samples was of least importance.

Table 5.1: Samples for Test 1 and 2
Table includes archaeological information, context type and extraction protocol. Country codes are: SW- Switzerland, GE- Germany, UK- United Kingdom, DK- Denmark, SP- Spain, PT- Portugal, CR- Czech Republic, MO- Morocco, IS- Israel, KZ- Kazakhstan.

Sample code	Country	Dating	Description	Context Type	Previous PCR work	Extraction Type	Used
SDBM 26-728	GE	c2800 BCE	Single burials from the Corded Ware culture	Single Burial soil samples	Yes	Flammer 2018	Test 1- Preliminary Shotgunning Test
SDBM 27-329	GE	c2800 BCE	Single burials from the Corded Ware culture	Single Burial soil samples	Yes	Flammer 2018	Test 1- Preliminary Shotgunning Test
ZPO 6625	SW	c3060 BCE	Neolithic stilt settlement/communal deposit	Communal soil samples	Yes	Flammer 2018	Test 1- Preliminary Shotgunning Test
ZPO 12156	SW	c3630 BCE	Neolithic stilt settlement/communal deposit	Communal soil samples	Yes	Flammer 2018	Test 1- Preliminary Shotgunning Test
HLGV AS27 4/5	GE	c1300 CE	House-associated latrine from the Merchant's quarter of Lübeck	Communal soil samples	Yes	Flammer 2018	Test 1- Preliminary Shotgunning Test
HLGV FS26 1/3	GE	c1625 CE	House-associated latrine from the Merchant's quarter of Lübeck	Communal soil samples	Yes	Flammer 2018	Test 1- Preliminary Shotgunning Test
BFR 1036-1156	UK	c1150 CE	Communal waste ditch outside city	Communal soil samples	Yes	Flammer 2018	Test 1- Preliminary Shotgunning Test
BFR 2038-2567	UK	c1500 CE	Communal waste ditch outside city	Communal soil samples	Yes	Flammer 2018	Test 1- Preliminary Shotgunning Test
Hg	UK	c900 CE	Anglo-Saxon cesspit	Communal soil samples	Yes	Flammer 2018	Test 1- Preliminary Shotgunning Test
VDKVS K301	DK	c1030 CE	Latrine sample associated with a Viking age longhouse	Communal soil samples	Yes	Flammer 2018	Test 1- Preliminary Shotgunning Test

Sample code	Country	Dating	Description	Context Type	Previous PCR work	Extraction Type	Used
VDKVS K304	DK	c1030 CE	Latrine sample associated with a Viking age longhouse	Communal soil samples	Yes	Flammer 2018	Test 1- Preliminary Shotgunning Test
PREDW1	CR	Palaeolithic	Pelvic sediment from a wolf	Single Burial soil samples in water	No	Haile 2012 Dabney 2013	Test 1- Preliminary Shotgunning Test
COLL28353-6	SP	Mesolithic	Pelvic sediment from human burial	Single Burial soil samples in water	No	Haile 2012 Dabney 2013	Test 1- Preliminary Shotgunning Test
COLL28350-5	SP	Mesolithic	Pelvic sediment from human burial	Single Burial soil samples in water	No	Haile 2012 Dabney 2013	Test 1- Preliminary Shotgunning Test
COVA2978	SP	Mesolithic	Pelvic sediment from human burial	Single Burial soil samples in water	No	Haile 2012 Dabney 2013	Test 1- Preliminary Shotgunning Test
TNB9621	UK	Mesolithic	Sediment near human burial	Soil sample in water	No	Haile 2012 Dabney 2013	Test 1- Preliminary Shotgunning Test
TB12534	UK	Mesolithic	Occupational Sediment from a midden	Communal soil samples in water	No	Haile 2012 Dabney 2013	Test 1- Preliminary Shotgunning Test
RHAF23264	MO	Palaeolithic	Occupational Sediment	Communal soil samples in water	No	Haile 2012 Dabney 2013	Test 1- Preliminary Shotgunning Test
CAM2014A62860	PT	Mesolithic	Occupational Sediment	Communal soil samples in water	No	Haile 2012 Dabney 2013	Test 1- Preliminary Shotgunning Test
RAQ10H18	IS	Palaeolithic	Occupational Sediment	Communal soil samples in water	No	Haile 2012 Dabney 2013	Test 1- Preliminary Shotgunning Test
TEM1	KZ	Bronze Age	Unknown animal Coprolite	Coprolite in water	No	Haile 2012 Dabney 2013	Test 1- Preliminary Shotgunning Test
ES14.1	SP	Palaeolithic	A coprolite 'supposedly Neanderthal'	Coprolite in water	No	Haile 2012 Dabney 2013	Test 1- Preliminary Shotgunning Test
ES14.2	SP	Palaeolithic	A coprolite 'supposedly Neanderthal'	Coprolite in water	No	Haile 2012 Dabney 2013	Test 1- Preliminary Shotgunning Test
EOMF	UK	Medieval	Midden Sediment	Communal soil samples	Yes	4 variants Flammer 2018	Test 2- Direct comparison shotgun and targeted PCR

Sample code	Country	Dating	Description	Context Type	Previous PCR work	Extraction Type	Used
DST	GE	6th c BCE	Chamber burial	Single Burial soil samples	No	4 variants Flammer 2018	Test 2- Direct comparison shotgun and targeted PCR
EL-4064	GE	700-1700	Medieval Burial	Single Burial soil samples	No	4 variants Flammer 2018	Test 2- Direct comparison shotgun and targeted PCR
EL-4017	GE	700-1700	Medieval Burial	Single Burial soil samples	No	4 variants Flammer 2018	Test 2- Direct comparison shotgun and targeted PCR
EL-4329	GE	700-1700	Medieval Burial	Single Burial soil samples	No	4 variants Flammer 2018	Test 2- Direct comparison shotgun and targeted PCR
EL-4155.1	GE	700-1700	Medieval Burial	Single Burial soil samples	No	4 variants Flammer 2018	Test 2- Direct comparison shotgun and targeted PCR
EL-4326	GE	700-1700	Medieval Burial	Single Burial soil samples	No	4 variants Flammer 2018	Test 2- Direct comparison shotgun and targeted PCR
EL-2442	GE	700-1700	Medieval Burial	Single Burial soil samples	No	4 variants Flammer 2018	Test 2- Direct comparison shotgun and targeted PCR

5.5 Test 1- Preliminary Shotgunning Test

5.5.1 Summary

The goal of this test was to establish the utility of shotgunning as a sequencing approach for the detection of helminths, without the large starting soil weight and floatation methodology used by Søe (2018). As a preliminary test, a mixture of sample types were selected: sediment from single burials (n=6), communal samples (n=14) and coprolites (n=3), see Table 5.1. Two sediment extraction protocols were identified that could be used, however 12 of the samples had a starting weight too low to be divided by two. Those 12 samples were extracted using the Haile (2012) methodology (TNB9621, TB12534, ES14.1, RHAF23264, PREDW1, COLL28353-6, COLL28350-5, CAM2014A62860, COVA2978, ES14.2, RAQ10H18, TEM1). As these samples arrived in water, the water was also extracted using an adaptation of the Dabney (2013) protocol to test for soluble DNA. The remaining 11 samples were extracted using the Flammer (2018) protocol (ZPO 6625, ZPO 12156, SDBM 26-728, SDBM 27-329, HLGV AS27 4/5, HLGV FS26 1/3, BFR 1036-1156, BFR 2038-2567, Hg, VDKVS K301, VDKVS K304). These 11 samples were also extracted using the Haile (2012) method however these runs were unsuccessful and excluded from this study (a discussion of these runs can be found in Supplementary 5.10.3).

5.5.2 Results and Discussion

The sediments extracted using the Haile (2012) protocol were lost through laboratory error and therefore were not analysed. Of the remaining extracts, different stringency measures had a notable impact on the number of positive hits recovered, as can be seen in Figure 5.1. This may reflect the reference database available for the study of palaeoparasitology. The database used here for identifying BLAST matches was constructed using both published genomes available in NCBI's GenBank and those available from Wormbase. As the Wormbase website includes genomes that are in a draft format and unpublished, there is the potential for microbial, host or other contaminant to remain in the database (Korhonen et al., 2016). In addition, historically full helminth genomes have been difficult to reconstruct due to complex repetitive regions, particularly for holocentric nematodes (Eccles et al., 2018), so some taxa and gene regions are not represented in the database. The hits recovered here with low support may therefore either be of the closest helminth taxa represented in the database or false positives. To account for this uncertainty, the three levels of data processing have been retained for discussion.

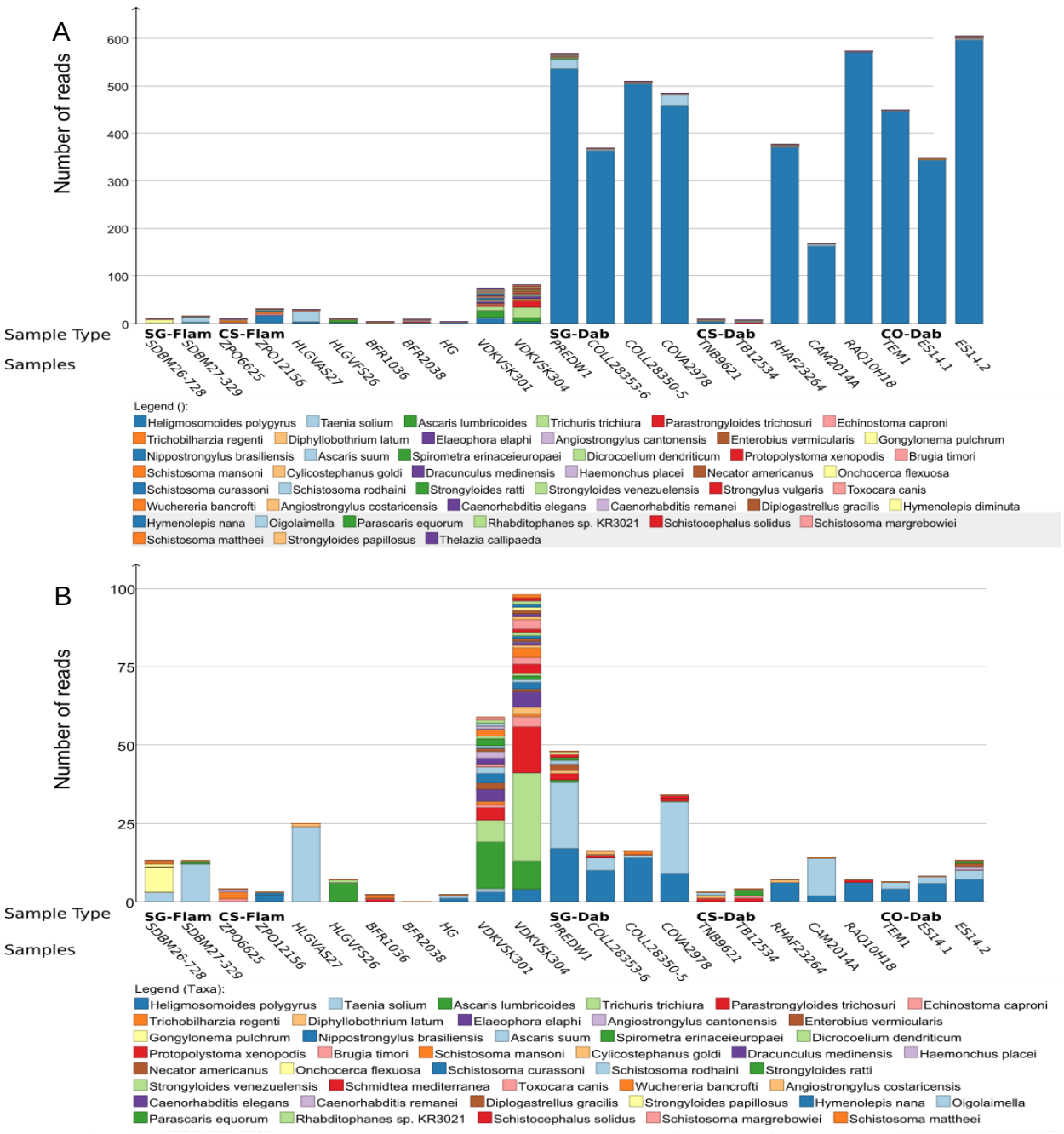

A
Number of reads
600
500
400
300
200
100
0
Sample Type
Samples
SG-Flam
CS-Flam
SG-Dab
CS-Dab
CO-Dab
SDBM26-728
SDBM27-329
ZPO6625
ZPO12156
HLGVIAS27
HLGVFS26
BFR1036
BFR2038
HG
VDKVSK301
VDKVSK304
PREDW1
COLL28353-6
COLL28350-5
COVA2978
TNB9621
TB12534
RHAF23264
CAM2014A
RAQ10H18
TEM1
ES14.1
ES14.2
Legend ():
Heligmosomoides polygyrus
Taenia solium
Ascaris lumbricoides
Trichuris trichiura
Parastrongyloides trichosuri
Echinostoma caproni
Trichobilharzia regenti
Diphyllobothrium latum
Elaeophora elaphi
Angiostrongylus cantonensis
Enterobius vermicularis
Gongylonema pulchrum
Nippostrongylus brasiliensis
Ascaris suum
Spirometra erinaceieuropaei
Dicrocoelium dendriticum
Protopolystoma xenopodis
Brugia timori
Schistosoma mansoni
Cylicostephanus goldi
Dracunculus medinensis
Haemonchus placei
Necator americanus
Onchocerca flexuosa
Schistosoma curassoni
Schistosoma rodhaini
Strongyloides ratti
Strongyloides venezuelensis
Strongylus vulgaris
Toxocara canis
Wuchereria bancrofti
Angiostrongylus costaricensis
Caenorhabditis elegans
Caenorhabditis remanei
Diplogastrellus gracilis
Hymenolepis diminuta
Hymenolepis nana
Oigolaimella
Parascaris equorum
Rhabditophanes sp. KR3021
Schistocephalus solidus
Schistosoma margrebowiei
Schistosoma mattheei
Strongyloides papillosus
Thelazia callipaeda
B
Number of reads
100
75
50
25
0
Sample Type
Samples
Legend (Taxa):
Schmidtea mediterranea
C
Number of reads
60
40
20
0
Helminths believed to be restricted to Animals- P. trichosuri (reads=5, sample 2), E. elaphi (reads=4,sample 2)
Gastrointestinal Human Helminths- T. solium (reads=90, sample=11), Ascaris spp. (reads=62, sample=4), T. trichiura (reads=32, sample=2)
Helminths that infect Humans but they are not the definitive host- G. pulchrum (reads=8, sample=1), S. erinaceieuropaei (reads=1, sample=1)

Figure 5.1: The results from Test 1 with different levels of data processing. Accronyms include: SG (Single Grave), CS (Communal Sample), CO (Corprolite), FLA (Flammer protocol) and DAB (Dabney protocol).
A- A bar plot showing the diversity of nematode and platyhelminth species detected in each sample. The size of the segments is indicative of the number of reads that matched to a species. Species are ordered by the most reads matching to the species, to the least.
B- The same graph as above however the data has been processed used UrQT for the removal of poly-A tails. A bar plot showing the diversity of nematode and platyhelminth species detected in each sample. Species are ordered in the same manner as Figure 1 with the exception of Schmidtea mediterranea which are novel to this round and Strongylus vulgaris, Hymenolepis diminuta and, Thelazia callipaeda which are no longer detected.
C- A bar plot showing the number of reads retained for taxa that are identified by more than one read in at least one sample and has an e-value stronger or equal to e-10. The taxa have been classified into three groups: helminths that have animals as their definitive hosts (P. trichosuri, E. elaphi), gastrointestinal helminths where humans are the definitive hosts (T. solium, Ascaris spp., T. trichiura) and finally helminths that can infect humans but they are not the definitive host (Gongylonema pulchrum, Spirometra erinaceieuropaei) to show what types of helminths are recovered reliably from these archaeological contexts.

The BLAST results recovered using the processing steps outlined in the methodology can be seen in Figure 5.1A. The number of reads per sample ranged between 7,342-936,307 (average 93,952). Of these, 4843 reads in total were identified as either nematode (4,650) or platyhelminth (193). 45 unique species of helminth matched to these reads but the most commonly identified helminth was *Heligmososmoides polygyrus*, which is an intestinal round worm of rodents which makes up 91.02% (n=4408) of all the platyhelminth and nematode hits. As can be seen in Figure 5.1A, the greatest quantity of *H. polygyrus* was found in the water samples extracted using the Dabney (2013) protocol (42 *H. polygyrus* hits for samples extracted using the Flammer (2018) methodology and 4366 hits for samples extracted using the Dabney (2013) methodology).

When the *H. polygyrus* hits were inspected by eye, the majority of the sequences had Poly-A tails at the ends. Many helminth genomes have been constructed using complimentary DNA (cDNA) libraries (Brindley et al., 2009) which utilise poly-A sites in messenger RNA. An overabundance of this homopolymer string could therefore be indicative that the libraries have not being cleaned for library construction by-product and potentially contamination. To ascertain the extent of this effect, the samples were run through a poly-A removal step using UrQT (Modolo and Lerat, 2015) and then reassessed.

After the poly-A tails had been trimmed, there was a drop to 576 reads identified as either nematode or platyhelminth. Whilst there was an overall drop, this coincided

with an increase to the number of reads identified as platyhelminth (n=229, an increase of 36). The biggest drop in hits were for samples extracted using the Dabney (2013) protocol, from 4,488 hits to 217, as can be seen in Figure 5.1B. 43 species of worm was identified, including 1 novel species (*Schmidtea mediterranea*) but this was not identified more than once. *Strongylus vulgaris, Hymenolepis diminuta* and, *Thelazia callipaeda* were no longer traced after the removal of poly-A tails. This time *Taenia solium* was the most abundant hit making up 21.18% (n=122) of the nematode and platyhelminth reads. *H. polygyrus* was still abundant, being the second most commonly identified helminth but made up 15.80% (n=91) of nematode and platyhelminth reads.

Whilst the number of helminth species detected was high, over half of them were only identified in a sample by one read. The low number of reads do not necessarily indicate that the helminth DNA was not present, but a single match meant that the identification of these species was not reliable. Table 5.2 lists the platyhelminth and nematode species that had more than one read identified in at least one sample (21 species). Nine of these were identified in more than one extract: *H. polygyrus, T. solium, Ascaris lumbricoides, Ascaris suum, Enterobius vermicularis, Parastrongyloides trichosuri, Elaeophora elaphi, T. trichiura* and, *Nippostrongylus brasiliensis*. For all species identified see Supplementary 5.10.4. As can be seen in Table 5.2 and Figure 5.1C, not all of these species had BLAST matches with an e-value support stronger than e-10. When this is accounted for, the remaining helminth species fall into three broad categories: helminths that have animals as their definitive hosts (*P. trichosuri, E. elaphi*), gastrointestinal helminths where humans are the definitive hosts *(T. solium, Ascaris* spp.*, T. trichiura)* and finally helminths that can infect humans but they are not the definitive host (*Gongylonema pulchrum*, *Spirometra erinaceieuropaei*).

As can be seen in Figure 5.1C, human gastrointestinal helminths make up the majority of the most robust BLAST matches (184 reads out 202). *T. solium* was recovered in the most samples (11) with a higher proportion of positive samples extracted using the Dabney (2013) protocol (8/12 samples) rather than the Flammer (2018) protocol (3/11). This could indicate that *T. solium* were easier to detect by biomolecular approaches when they are separated from a sedimentary context. *Ascaris* spp. meanwhile was the species of helminth that had the highest number of matches in a single sample (47). The higher recovery of human gastrointestinal helminths could reflect a higher proportion of human specific helminths in the reference database (due to their medical significance), or the fact that we would expect a higher proportion of

Table 5.2: Results of Test 1 after the removal of Poly-A tails.
A table summarising the shotgun reads after the Poly-A removal step and the type of BLAST matches identified. Platyhelminth and Nematode hits represent the number of reads that fell within this class, regardless of the specificity of the hit. The Number of helminth species was the number of taxa identified down to the species level. Taxa are only listed if 2 or more unique reads matched to the species to retain only the most robust hits and it is indicated the number of reads that are retained if the e-values is e-10≥.

Sample	Extraction Type	No. of trimmed reads	% Trimmed bases	Eukaryote Hits	Platyhelminth hits	Nematode hits	No. of helminth species	Species identified by 2< hits
SDBM 26-728	Flammer 2018	21867	2.11%	1440	3	55	4	*Taenia solium* (n=3;e10≥=3), *Gongylonema pulchrum* (n=8;e10≥=8)
SDBM 27-329	Flammer 2018	23832	1.51%	1126	12	1	2	*Taenia solium* (n=12;e10≥=11)
ZPO 6625	Flammer 2018	7628	1.90%	480	4	2	3	*Trichobilharzia regenti* (n=3;e10≥=0)
ZPO 12156	Flammer 2018	4400	3.38%	779	34	3	2	*Heligmosomoides polygyrus* (n=2;e10≥=0)
HLGV AS27 4/5	Flammer 2018	3397	4.74%	1692	26	0	2	*Taenia solium* (n=24;e10≥=20)
HLGV FS26 1/3	Flammer 2018	5116	2.10%	296	2	6	2	*Ascaris* spp. (n=6;e10≥=5)
BFR 1036-1156	Flammer 2018	18809	1.93%	757	1	1	2	
BFR 2038-2567	Flammer 2018	10722	1.88%	644	1	0	0	
Hg	Flammer 2018	6448	2.45%	691	1	1	2	

Sample	Extraction Type	No. of trimmed reads	% Trimmed bases	Eukaryote Hits	Platyhelminth hits	Nematode hits	No. of helminth species	Species identified by 2< hits
VDKVSK301	Flammer 2018	143324	1.74%	2062	5	93	23	*Ascaris* spp. (n=50;e10≥=47), *Enterobius vermicularis* (n=2;e10≥=0), *Parastrongyloides trichosuri* (n=5;e10≥=3), *Haemonchus placei* (n=2;e10≥=0), *Nippostrongylus brasiliensis* (n=3;e10≥=0), *Heligmosomoides polygyrus* (n=3;e10≥=0), *Dracunculus medinensis* (n=2;e10≥=0), *Elaeophora elaphi* (n=5;e10≥=3), *Wuchereria bancrofti* (n=2;e10≥=0), *Trichuris trichiura* (n=7;e10≥=6)
VDKVSK304	Flammer 2018	373947	1.88%	2511	21	87	31	*Trichuris trichiura* (n=29;e10≥=26), *Elaeophora elaphi* (n=5;e10≥=1), *Brugia timori* (n=2;e10≥=0), *Heligmosomoides polygyrus* (n=4;e10≥=0), *Nippostrongylus brasiliensis* (n=2;e10≥=0), *Parastongyloides trichosuri* (n=15;e10≥=5), *Toxocara canis* (n=3;e10≥=0), *Ascaris* spp. (n=14;e10≥=9), *Schistosoma mansoni* (n=3;e10≥=0), *Echinostoma caproni* (n=3;e10≥=0), *Protopolystoma xenopodis* (n=3;e10≥=0), *Diphyllobothrium latum* (n=2;e10≥=0)
PREDW1	Dabney (2013)	29833	1.88%	2774	25	25	11	*Taenia solium* (n=22,e10≥=18), *Ascaris* spp. (n=2,e10≥=1), *Enterobius vermicularis* (n=2,e10≥=0), *Parastrongyloides trichosuri* (n=2,e10≥=0), *Heligmosomoides polygyrus* (n=17,e10≥=0)
COLL28353-6	Dabney (2013)	23201	1.44%	853	4	12	4	*Heligmosomoides polygyrus* (n=10, e10≥=0), *Taenia solium* (n=4, e10≥=1)
COLL28350-5	Dabney (2013)	4389	2.59%	655	2	14	3	*Heligmosomoides polygyrus* (n=14;e10≥=0)
COVA2978	Dabney (2013)	26410	1.62%	1954	25	10	4	*Heligmosomoides polygyrus* (n=9;e10≥=0), *Taenia solium* (n=23;e10≥=12)

Sample	Extraction Type	No. of trimmed reads	% Trimmed bases	Eukaryote Hits	Platyhelminth hits	Nematode hits	No. of helminth species	Species identified by 2< hits
TNB9621	Dabney (2013)	11632	2.04%	602	2	1	3	
TB12534	Dabney (2013)	15230	2.22%	1971	17	2	5	*Taenia solium* (n=12;e10≥=11), *Spirometra erinaceieuropaei* (n=2;e10≥=1)
RHAF23264	Dabney (2013)	4488	2.12%	674	22	6	2	*Heligmosomoides polygyrus* (n=6;e10≥=0)
CAM2014 A62860	Dabney (2013)	21808	1.68%	876	12	2	2	*Heligmosomoides polygyrus* (n=2;e10≥=0), *Taenia solium* (n=12;e10≥=9)
RAQ10H18	Dabney (2013)	10642	2.07%	975	0	7	2	*Heligmosomoides polygyrus* (n=6;e10≥=0)
TEM1	Dabney (2013)	5754	2.09%	632	3	4	2	*Heligmosomoides polygyrus* (n=4;e10≥=0), *Taenia solium* (n=2;e10≥=1)
ES14.1	Dabney (2013)	3651	2.35%	569	2	6	2	*Heligmosomoides polygyrus* (n=6;e10≥=0), *Taenia solium* (n=2;e10≥=1)
ES14.2	Dabney (2013)	13548	2.00%	999	5	9	5	*Heligmosomoides polygyrus* (n=7;e10≥=0), *Taenia solium* (n=3;e10≥=3)

human gastrointestinal helminths in these contexts. The two exception are samples PREDW1 and TEM1 which both come from an animal context. Both samples had reads matching to *T. solium* and PREDW1 had a read matching to *Ascaris* spp.. Both of these helminth species could be indicative of the consumption of pig (as *A. suum* is not clearly distinct from *A. lumbricoides* genetically), even if the worms could not develop in these hosts.

The two helminth types that were identified as non-human (*P. trichosuri, E. elaphi*) were found in samples VDKVS K301 and VDKVS K304 which are both latrine samples associated with a Viking Age Long house from Viborg Søndersø, Denmark. Communal latrines and pits that are open could be contributed to by animals, however the two species identified here are restricted to regions that differ from the site (*P. trichosuri* are found in Australian Brush-tailed possums (Grant et al., 2006)*, E. elaphi* are found in Iberian red deer in the Southern and Central Iberian Peninsula (Höfle et al., 2004)). The two helminth types that can accidently infect human hosts (*G. pulchrum*, *S. erinaceieuropaei*) would not be expected in a communal latrine as humans would not release these helminth eggs with their faeces. The two samples that contained these types of helminth were however, from single grave contexts which may contain traces of worms that would be located in subcutaneous tissue (*S. erinaceieuropaei)* or the oesophagus (*G. pulchrum*). These accidental infections are quite rare in the modern literature with only 52 cases of infection by *G. pulchrum* between the late 19th century and 2005 (Haruki et al., 2005) and 300 cases on record for spargonosis, caused by *Spirometra* spp., on record (Pampiglione et al., 2003).

The samples with the most species identified, and the most strongly supported nematode and platyhelminth reads were samples VDKVS K301 and VDKVS K304. These samples also differed from the rest by the number of hits to *Ascaris* spp. and *T. trichiura*. For samples VDKVS K301, there were 50 reads (e10≥=47) aligning to *Ascaris* spp. and 7 (e10≥=6) to *T. trichiura,* whilst for samples VDKVS K304 there were 14 hits (e10≥=9) to *Ascaris* spp. and 29 hits (e10≥=26) to *T. trichiura*. In the rest of the samples, only 4 had hits to *Ascaris* spp. (6 or fewer) but none had hits to *T. trichiura.* Samples: SDBM 26-728, SDBM 27-329, ZPO 6625, ZPO 12156, HLGV AS27 4/5, HLGV FS26 1/3, BFR 1036-1156, BFR 2038-2567, Hg, VDKVS K301, VDKVS K304 were selected due to the high number of *Ascaris* spp. and *T. trichiura* eggs that were identified by microscopy and the high number of reads recovered during targeted PCR. It was originally hypothesised that the results of samples VDKVS K301 and VDKVS K304 would also be replicated in the other samples.

5.5.3 Concluding comments from preliminary shotgunning tests

This preliminary run has shown that multiple helminth species can be identified using a shotgunning approach, without the use of large amounts of starting material and floatation as in Søe et al. (2018). Whilst some of the hits may reflect the incomplete nature of the reference databases, this issue is only going to reduce over time and make this approach for palaeoparasitology more reliable. As can be seen from samples extracted using the Flammer (2018) methodology however, this approach is not necessarily capable of identifying all helminths in these contexts as some taxa that were clearly identified by microscopy were not identified using this genetic approach. This may be indicative that the new extractions did not produce the same amount of endogenous genetic material. Alternatively, the shotgunning approach may not be sensitive enough to identify all helminths present either from the inhibiting effects of the soil matrix or from the data processing steps which have not been specifically adapted for palaeoparasitology. To test whether the lack of hits is due to a lack of genetic helminth material in the extracts, a direct comparison of targeted PCR and shotgunning was selected for the next test.

5.6 Test 2- Direct comparison of shotgunning and targeted PCR

5.6.1 Summary

The goal of this test was to identify whether shotgun sequencing identified more of less samples containing endogenous helminth DNA, with a focus on *Ascaris* spp., than the established targeted PCR approach. The second test was run on eight new soil samples, see Table 5.1. Seven of these samples were from burials (DST, EL-4064, EL-4017, EL-4329, EL-4155.1, EL-4326, EL-2442) that had previously been analysed by microscopy (samples EL-2442 and EL-4326 were identified as negative samples). The communal sample EOMF was included as a positive control. Each sample was extracted using 4 variants on the Flammer (2018) protocol as described in the methodology (FK- DNeasy Mericon Food kit, BK- Qiagen DNeasy Blood and Tissue kit, KIT- kit column, MIN- Qiagen MinElute). The extracts and blanks were then run through shotgun sequencing and targeted PCR. Four of the primers targeted different helminths (*Ascaris* spp., *T. trichiura, T. solium* and *T. saginata*) whilst two of the primers were used to indicate PCR inhibition (universal Eukaryote mitochondrial 16S and cryptosporidium 18S).

5.6.2 Results and Discussion

5.6.2.1 Results-Gel run

Of the first round of PCR, there were no successful bands for the cryptosporidium or either of the *Taenia* targets however, there were some bands for the universal 16S,

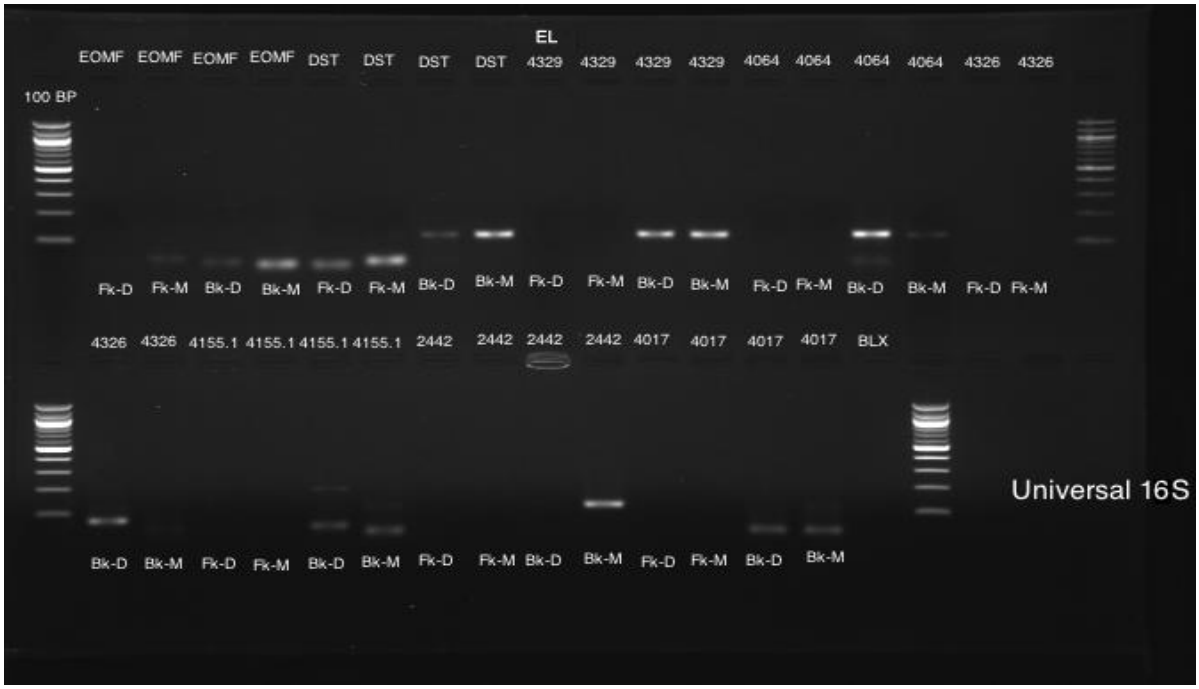


Figure 5.2: An image of the gel run from Test 2 using the universal 16S primer developed by Horreo et al. (2013). A 100 bp ladder was used. The labels at the top indicate the sample, and the labels at the bottom indicate the extraction type.

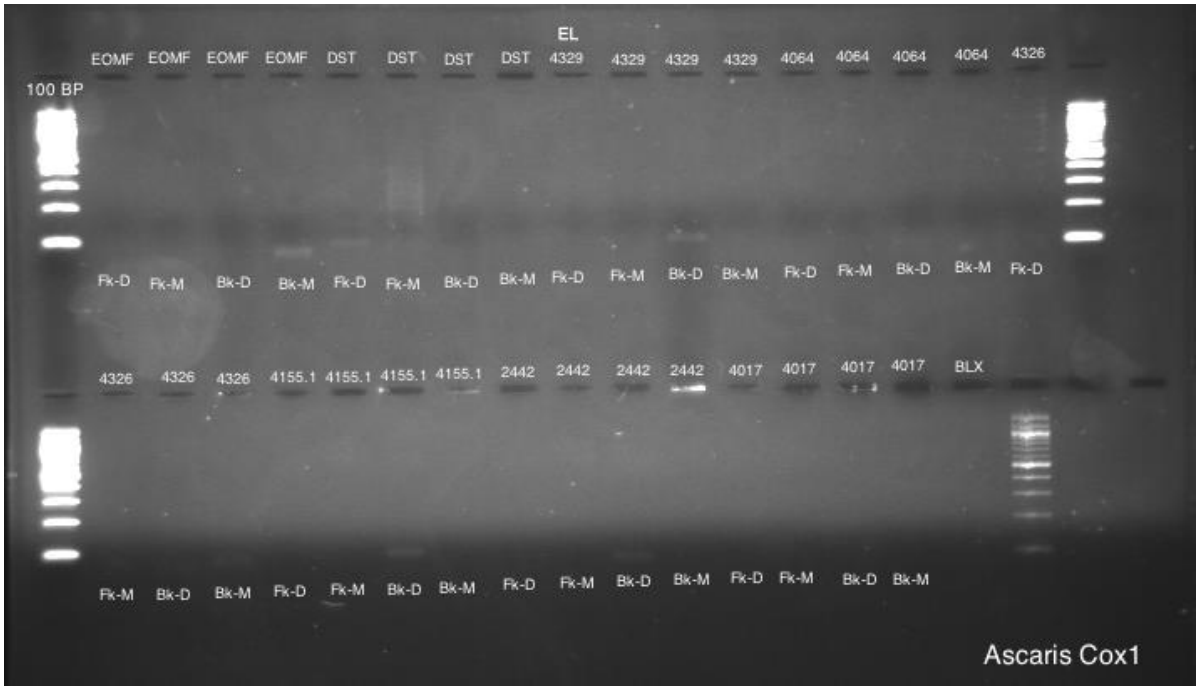


Figure 5.3: An image of the gel run from Test 2 using the Ascaris lumbricoides CO1 (241 bp) targets developed by Flammer (2014). A 100 bp ladder was used. The labels at the top indicate the sample, and the labels at the bottom indicate the extraction type.

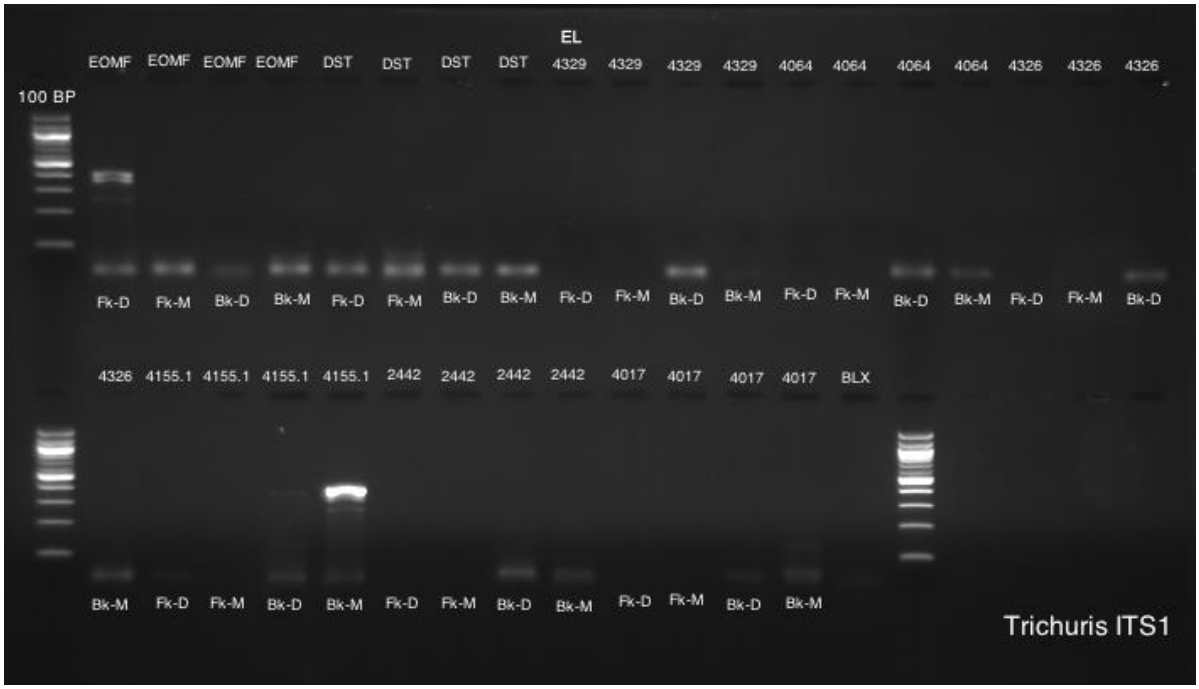


Figure 5.4: An image of the gel run from Test 2 using the T. trichiura ITS1 (459 bp) targets developed by Flammer (2018). A 100 bp ladder was used. The labels at the top indicate the sample, and the labels at the bottom indicate the extraction type.

Ascaris spp. and *Trichuris* targets although the bands for *Ascaris* spp. were very faint (Figures 5.2, 5.3 and 5.4). In the universal target gel, we can see that for 7 of the samples the Blood and Tissue kit extractions were the only ones to produce bands but, this pattern isn't replicated for the 2 other targets. There was no consistent difference detected when comparing the 2 column types. Of the 192 first round PCR runs, 31 were selected for library build and sequencing. After the library build, the bands became much more distinct (see Figure 5.5). For the *Ascaris* spp. and *T. trichiura* library builds the bands produced were at the right length and for the eukaryote primer samples there was a band consistently around 150-170 bp level (except EOMF). Whilst there is contamination in the blanks for both the *Ascaris* spp. and *T. trichiura* run, these bands are not the same length as the archaeological bands. As multiple bands of various lengths were noted in the *T. trichiura* run, these were submitted for sequencing to assess whether off target amplification had occurred.

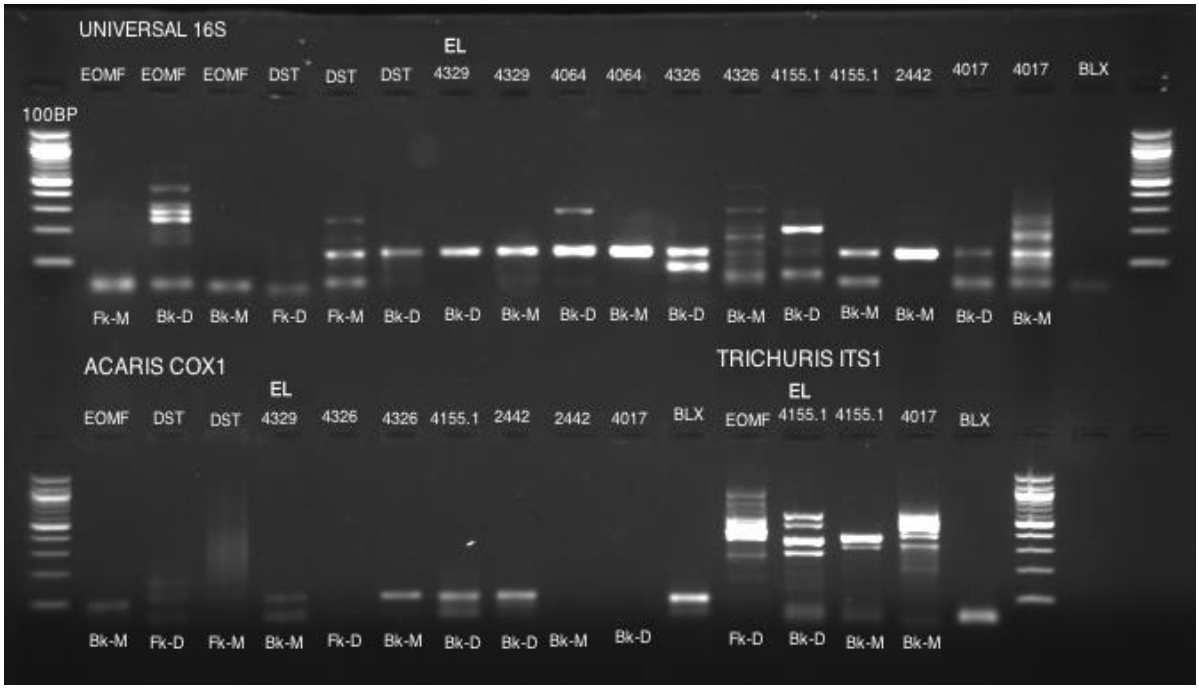


Figure 5.5: An image of the gel run from Test 2 of the samples selected for sequencing post library building. A 100 bp ladder was used. The labels at the top indicate the sample, and the labels at the bottom indicate the extraction type and the primers used are indicated at the start of each group.

5.6.2.2 Results- Targeted approach, BWA

Ten samples were submitted for sequencing that were amplified using targeted *Ascaris* spp. primers, and all ten had positive matches to an *A. lumbricoides* reference full mitochondria (accession code HQ704900.1). For two of the samples (EOMF-BK-MIN and EL4329-BK-MIN), the aligning region was 35-50 bp long but the entire reads were 153-248 bp. Whilst these short reads did not just align to the primer region, they also did not reflect the target region so were classified as unsuccessful. Of the remaining samples with reads in the right loci, three of them (DST-FK-KIT, EL4155.1-BK-KIT, EL2442-BK-KIT) only had one read which may either indicate a low quantity of endogenous DNA or contamination. The two samples with the highest number of aligning reads were from burials, the pot burial (DST-FK-MIN- 37X) and from a single grave sample (EL4326-FK-KIT- 33X). The other positive samples had 13 aligning reads or less (EL4017-BK-KIT 3X, EL4326-BK-MIN 9X, EL2442-BK-MIN 13X). Whilst the reads show a degree of variation, these are random point mutations that are not found in more than one read. As has been found in other papers (such as Iñiguez et al., 2006), the samples that were negative for microscopy (4326 and 2442) were positive for *Ascaris* spp. reads in at least one of the extraction types. There was no relation between extraction type used and positive results. For a summary of the results see Table 5.3. Of the *T. trichiura* samples submitted for sequencing, none of them produced reads that aligned to a reference *T. trichiura* ITS1 gene (accession code GQ352552.1).

Table 5.3: A table summarising which samples produced endogenous reads for each sequencing approach
Codes
TPCR- Sent for sequencing after targeted PCR using primers either for Ascaris CO1 (ASC), Trichurs ITS1 (TRI) or universal 16S (UNI)
BWA- alignment using the BWA tool kit to a reference genome for an Ascaris full mitochondrial (ASC- accession code HQ704900.1) or a Trichuris ITS1 gene (TRI- accession code GQ352552.1)
UNI BLAST ASC- The reads recovered from the TPCR using the universal 16S primers were run through BLAST after dereplication to identify which samples had reads that were identified as Ascaris.
Shot- BLAST ASC- the number of hits from the shotgunning runs to Ascaris using BLAST.
Short read- The off target hit which was identified in a number of runs (sequence: TTGGTATGGATCTTGATTCTCGGGCTTATTTTACTGCT)

Sample + Extraction	TPCR ASC	BWA ASC	TPCR TRI	BWA TRI	TPCR UNI	BWA ASC	UNI-BLAST ASC	Shot-BLAST ASC
EOMF-FK-MIN					Y	0		
EOMF-FK-KIT			Y	0				
EOMF-BK-MIN	Y	Short read			Y	Short read		
EOMF-BK-KIT					Y	Short read		
DST-FK-MIN	Y	x37			Y	Short read	Short read	
DST-FK-KIT	Y	x1			Y	0		2
DST-BK-MIN								
DST-BK-KIT					Y	Short read		
EL4064-FK-MIN								
EL4064-FK-KIT								
EL4064-BK-MIN					y	Short read		
EL4064-BK-KIT					Y	Short read		
EL4017-FK-MIN								
EL4017-FK-KIT								
EL4017-BK-MIN			Y	0	Y	Short read	Short read	
EL4017-BK-KIT	Y	x3			Y	Short read		
EL4329-FK-MIN								
EL4329-FK-KIT								

EL4329-BK-MIN	Y	Short read			Y	Short read		
EL4329-BK-KIT					Y	Short read		
EL4155.1-FK-MIN								
EL4155.1-FK-KIT								
EL4155.1-BK-MIN			Y	0	Y	Short read	Short read	
EL4155.1-BK-KIT	Y	x1	Y	0	Y	Short read	Short read	
EL4326-FK-MIN								
EL4326-FK-KIT	Y	x33						
EL4326-BK-MIN	Y	x9			Y	Short read		
EL4326-BK-KIT					Y	Short read		1
EL2442-FK-MIN								
EL2442-FK-KIT								
EL2442-BK-MIN	Y	x13			Y	Short read		3
EL2442-BK-KIT	Y	x1						

The universal 16S samples were also aligned to a reference mitochondrion for both *T. trichiura* (accession code AP017704.1) and *A. lumbricoides*. Whilst there were no alignments to the *T. trichiura* reference genome, 15 samples had positive alignments with *A. lumbricoides*. Rather than aligning to the 16S region, the reads aligned to the COX1 gene, in the same region as the short erroneous reads for the *Ascaris* spp. specific primers. Again, the aligning region (35-48 bp) is not the same length as the total read (183-462 bp). To ascertain whether this region was just highly conserved and shared between multiple organisms, the aligning region (sequence TTGGTATGGATCTTGATTCTCGGGCTTATTTTACTGCT) was run through BLAST. The top 100 hits were all *Ascaris* sp. with an E values between 3e-08 and 5e-10. The *Ascaris spp.* hits with the 16S targets are not as robust as those found using the species-specific primers but it is worth noting that 5 of these extracts were also positive for *Ascaris* spp. using *Ascaris* spp. specific targets.

When the 16S reads were run through BLAST rather than aligning to a reference genome, 4 of the 18 samples still retained hits for *Ascaris* spp. (DST-FK-MIN, EL4017-BK-MIN, EL4155.1-BK-MIN, EL4155.1-BK-KIT). Three out of four of these samples were

extracted using a MinElute rather than the kit column and of the one successful case where parasites were detected using a kit column, there was a greater number of fragments matching to *Ascaris* spp. when the same sample was extracted using a MinElute column (3 rather than 1). The fragment matches were the same short region identified in the alignments of the targeted *Ascaris* and universal 16S read to the *A. lumbricoides* reference genome.

5.6.2.3 Results- Shotgunning, BLAST

13 of the samples had no nematode or platyhelminth species identified but of these, 8 samples had reads that were identified as generic platyhelminth (i.e. reads matched equally to a number of different platyhelminth species). These generic hits are likely to represent PCR by-product matching to the incomplete helminth database rather than true endogenous hits, as 10 samples had generic platyhelminth hits making up at least half of all the Eukaryote hits (66.67-93.33% of reads, average 85.96%). Considering the mixed context of soil samples, this is unlikely to be a true reflection of the genetic content of these samples. Of the samples with species specific hits, 34 unique species of helminth were identified and 13 of these taxa had more than one read assigned to them in at least one sample. For a summary of these results see Table 5.4.

Sample EL2442-BK-MIN had the highest number of helminth taxa identified by BLAST, with the majority of reads matching to *E. elaphi*. As was found in Test 1, there is a disconnect between the region that *E. elaphi* are restricted to (Southern and Central Iberian Peninsula) and the location of the site (Germany) which may reflect a database problem for this species. In three samples (DST-FK-KIT, EL4326-BK-KIT, EL2442-BK-MN), *Ascaris* spp. were identified although the number of hits were low (maximum 3). For these three samples, *Ascaris* spp. hits were also identified by the targeted PCR approach (the *Ascaris* spp. specific and/or Eukaryote 16S primers). This is summarised in Table 5.3. Again, none of the extraction protocols were consistently stronger for producing either a high number of total reads or identifying endogenous helminth reads suggesting that sediment type may be a more important factor.

Table 5.4: Results of Test 2
A table summarising the shotgunning run in the Target PCR vs Shotgunning test and the type of BLAST matches identified. Platyhelminth and Nematode hits represent the number of reads that fell within this class, regardless of the specificity of the hit. The Number of helminth species was the number of taxa identified down to the species level. Taxa are only listed if 2 or more unique reads matched to the species to retain only the most robust hits, and the number of reads that are retained if the e-values is e-10≥ is indicated.

Sample + extraction	No. of trimmed reads	% Trimmed bases	Eukaryote Hits	Platyhelminth hits	Nematode hits	No. of helminth species	Species identified by 2< hits
EOMF-FK-MIN							
EOMF-FK-KIT	16325	1.43%	276	4	4	6	
EOMF-BK-MIN	6899	2.06%	334	4	2	3	
EOMF-BK-KIT	856	5.15%	385	0	3	1	*Heligmosomoides polygyrus* (n=3;e10≥=0)
DST-FK-MIN	3011	2.04%	251	2	2	1	*Heligmosomoides polygyrus* (n=2;e10≥=0)
DST-FK-KIT	65944	1.65%	2869	30	17	14	*Diphyllobothrium latum* (n=2;e10≥=0), *Spirometra erinaceieuropaei* (n=4;e10≥=1), *Echinostoma caproni* (n=3;e10≥=0), *Schistosoma japonicum* (n=3;e10≥=0), *Ascaris* spp. (n=2;e10≥=2), *Heligmosomoides polygyrus* (n=7;e10≥=0), *Elaeophora elaphi* (n=3;e10≥=0)
DST-BK-MIN	1427	2.82%	324	289	0	0	
DST-BK-KIT	662	4.70%	225	210	0	1	
EL4064-FK-MIN	1037	6.34%	535	461	0	0	
EL4064-FK-KIT	54	2.51%	2	0	0	0	

Sample + extraction	No. of trimmed reads	% Trimmed bases	Eukaryote Hits	Platyhelminth hits	Nematode hits	No. of helminth species	Species identified by 2< hits
EL4064-BK-MIN	74	7.01%	40	34	0	0	
EL4064-BK-KIT	347	3.59%	103	90	0	0	
EL4017-FK-MIN	1664	4.56%	1100	232	1	1	
EL4017-FK-KIT	355	8.68%	242	221	0	0	
EL4017-BK-MIN	285	2.43%	53	47	0	0	
EL4017-BK-KIT	9272	1.48%	470	380	1	1	
EL4329-FK-MIN	170	3.15%	46	4	0	0	
EL4329-FK-KIT	634	3.73%	201	134	2	3	
EL4329-BK-MIN	183	2.43%	28	25	0	0	
EL4329-BK-KIT	972	3.20%	35	0	0	0	
EL4155.1-FK-MIN	526	5.08%	37	16	1	1	
EL4155.1-FK-KIT	44099	1.22%	283	1	3	2	
EL4155.1-BK-MIN	100	1.54%	3	0	0	0	
EL4155.1-BK-KIT	87	3.33%	4	0	0	0	
EL4326-FK-MIN	186	3.31%	10	1	0	1	

Sample + extraction	No. of trimmed reads	% Trimmed bases	Eukaryote Hits	Platyhelminth hits	Nematode hits	No. of helminth species	Species identified by 2< hits
EL4326-FK-KIT	1044	5.23%	64	1	1	2	
EL4326-BK-MIN	529	4.18%	23	1	1	1	
EL4326-BK-KIT	31143	1.20%	798	3	5	6	*Elaeophora elaphi* (n=2;e10≥=1)
EL2442-FK-MIN	958	7.18%	28	1	1	1	
EL2442-FK-KIT	249	6.33%	10	0	0	0	
EL2442-BK-MIN	554219	1.13%	3329	10	112	22	*Brugia timori* (n=3;e10≥=2), *Elaeophora elaphi* (n=77;e10≥=71), *Heligmosomoides polygyrus* (n=4;e10≥=0), *Nippostrongylus brasiliensis* (n=2;e10≥=0), *Caenorhabditis briggsae* (n=2;e10≥=0), *Parastrongyloides trichosuri* (n=6;e10≥=3), *Toxocara canis* (n=2;e10≥=0), *Parascaris equorum* (n=3;e10≥=0), *Ascaris* spp. (n=3;e10≥=2), *Echinostoma caproni* (n=2;e10≥=0), *Spirometra erinaceieuropaei* (n=4;e10≥=1)
EL2442-BK-KIT	29614	1.14%	235	4	7	6	*Elaeophora elaphi* (n=4;e10≥=1)

5.6.3 Concluding comments from shotgunning vs targeted PCR test

The key conclusion of these results is that the targeted approach identified more positive samples that contained endogenous *Ascaris* spp. DNA than a shotgunning approach when the same extracts were used. Positive identification of *Ascaris* spp. occurred three times using the shotgunning protocol whilst 8 samples were identified as positive using *Ascaris* spp. specific primers (out of 32 extracts) and of these, 5 had a read depth greater than one. Whilst it is not necessarily surprising that the depth of reads is greatest for targeted PCR, there is the argument that shotgunning could be better at identifying samples with endogenous *Ascaris* spp. DNA as there is the capacity to amplify any genomic region that is preserved, not just the region of choice. From these results, we can see that this is not the case. Instead, the power of detection may be reduced by the preferential amplification of environmental DNA over the ancient helminth DNA which suggests that deeper sequencing may be required for the shotgunning approach to palaeoparasitology. Alternatively, the power to identify multiple gene regions may be mitigated by the incomplete nature of the reference database. Whilst this shotgunning approach can identify multiple species of helminth from single grave samples, we have also established that it will not identify all endogenous helminth species. This suggests that if a researcher is interested in tracing a single species, shotgunning is not appropriate as a screening tool.

5.7 Test 3- Improving the recovery of variable genetic regions

5.7.1 *Ascaris* spp. target development and testing

Test 1 and 2 have established that a targeted PCR approach is currently the most sensitive to endogenous helminth DNA in sedimentary contexts. The next step for the analysis of population dynamics of the *Ascaris* spp. detected in Chapter 3 is therefore to develop primers that target variable regions. Whilst *A. lumbricoides* primers have been designed prior to this study for investigating archaeological parasites (Leles et al., 2008; Loreille et al., 2001), they have been designed for the confirmation of microscopic identification. Novel primers therefore needed to be developed. The degree of variation that can be recovered from archaeological samples and the reliability of the technique will show whether the methods developed here are suitable for the wide scale analysis of single graves in the UK.

5.7.2 Identifying targets of interest

To begin, a literature review of papers assessing genetic variation in modern *A. lumbricoides* was undertaken to identify genetic regions of utility. Four were identified: COX1 (Anderson and Jaenike, 1997; Betson et al., 2011, 2012, 2014; Cavallero et al., 2011; Iñiguez et al., 2012; Peng et al., 2005; Snábel et al., 2012; Zhou et al., 2011), NAD1 (Iñiguez et al., 2012; Peng et al., 2005; Zhou et al., 2011), ITS1 (Cavallero et al.,

2011; Das et al., 2015; Leles et al., 2010; Palma et al., 2019; Snábel et al., 2012; Sow et al., 2017) and microsatellites (Betson et al., 2014; Criscione et al., 2007). NAD1 was excluded from further consideration as a low number of modern studies utilised this region. There were also only a few papers utilising microsatellites but two of these found structural genetic diversity (Betson et al., 2011; Criscione et al., 2007). Unfortunately, the difficulty with microsatellite targets in ancient samples is that all of the same targets would need to work consistently. Considering the degradation of DNA that occurs in ancient samples, it is likely that the comparative power of microsatellites would be diminished for ancient samples in comparison to modern studies.

This left COX1 and ITS1. At the time of this study 61 COX1 and 78 ITS1 genes were available from NCBI for comparison (October 2017). Both have successfully been used to identify a number of haplotypes, and the papers on the COX1 region have consistently detected three distinct clades. Whilst there is debate on the significance of clades A & B, clade C exclusively represents European pigs (Nejsum et al., 2017). The shortest targets identified in the modern literature for each of the gene regions was included in this study (COX1-384-450 BP (Peng et al., 2005); ITS1- 87bp (Wiria et al., 2010)).

Unfortunately, the majority of targets utilised in the modern studies were long (312-1000 bp), therefore shorter targets needed to be designed as they are better adapted for the recovering of fragmented aDNA. Published sequences for the COX1 and ITS1 regions in *Ascaris* spp. were downloaded from NCBI and aligned in Geneious (Kearse et al., 2012). Published primers found in the literature review were then aligned to this collection of sequences to indicate regions of variability before fragments between 100-200 bp long were selected by visual assessment. This was particularly problematic for the COX1 gene as many of the variable regions were not interspersed by conservative sites suitable for primer attachment.

As the number of modern studies looking at the regional genetic variation of helminths was low and limited to a small number of target regions, studies of full mitochondria were also reviewed to identify other targets. Nejsum et al.'s (2017) study of complete mitochondria from *Ascaris* spp. found that the three clades identified using the COX1 gene were maintained in phylogenetic analysis of complete mitochondria. Further to this, they identified almost 800 SNPs which were used as the basis for additional primer design.

First the list was reduced to focus only on SNPs that occurred in sequences identified as *Ascaris lumbricoides* to ensure, despite the debate, that the variation detected was not a signifier of species differences. SNPs that fell within 100bp of each other were then identified as target regions for maximum variation. To assess which of these variants formed monophyletic groups or could be used for distinguishing individuals, the SNPs were mapped against a phylogenetic tree of all published complete *Ascaris* spp. mitochondria using Mesquite (Maddison and Maddison, 2018). SNP groups that could distinguish different clades and branches on the phylogenetic tree were selected for further processing (see Figure 5.6 for an example of the degree of variation in the SNPs using primer ASC_ND2_F1). In addition to this, individual SNPs that could be used to separate the A and B clade were also investigated as target regions. These target regions were kept short (100 bp or under, primers included) to increase the likelihood of working on fragmented aDNA, whilst retaining the capacity to discern the proportion of A and B sequences in a site. These targets could therefore be used to trace whether there was any shift in the proportions of these well-established haplogroups over time.

Sequences including the target areas identified from the SNP analysis was run through Primer3 (Untergasser et al., 2012) to identify the optimum area for annealing primers. For both the grouped and individual SNPs, the 100 bp sequences prior and post to the regions of interest were included to ensure the primer placement was not within the target areas. Where possible a high TM was selected but in other cases suitable primers could not be identified. Once the primers were designed, they were run through the PCR Primer Stats program (Stothard, 2000) to check for primer dimer, self-annealing, hairpin formation, TM and GC clamp. Cross primer dimer was then checked for using the ThermoFisher Multiple Primer Analyzer (ThermoFisher Scientific, 2018). The primer regions were then assessed by eye to check whether the 3 Prime end was conserved across the 19 published full mitochondria and for the level of conservation in the primers. Finally, the primers were run through blast to check the specificity of the primers. Primers that were identified as problematic using these methods were excluded from further consideration.

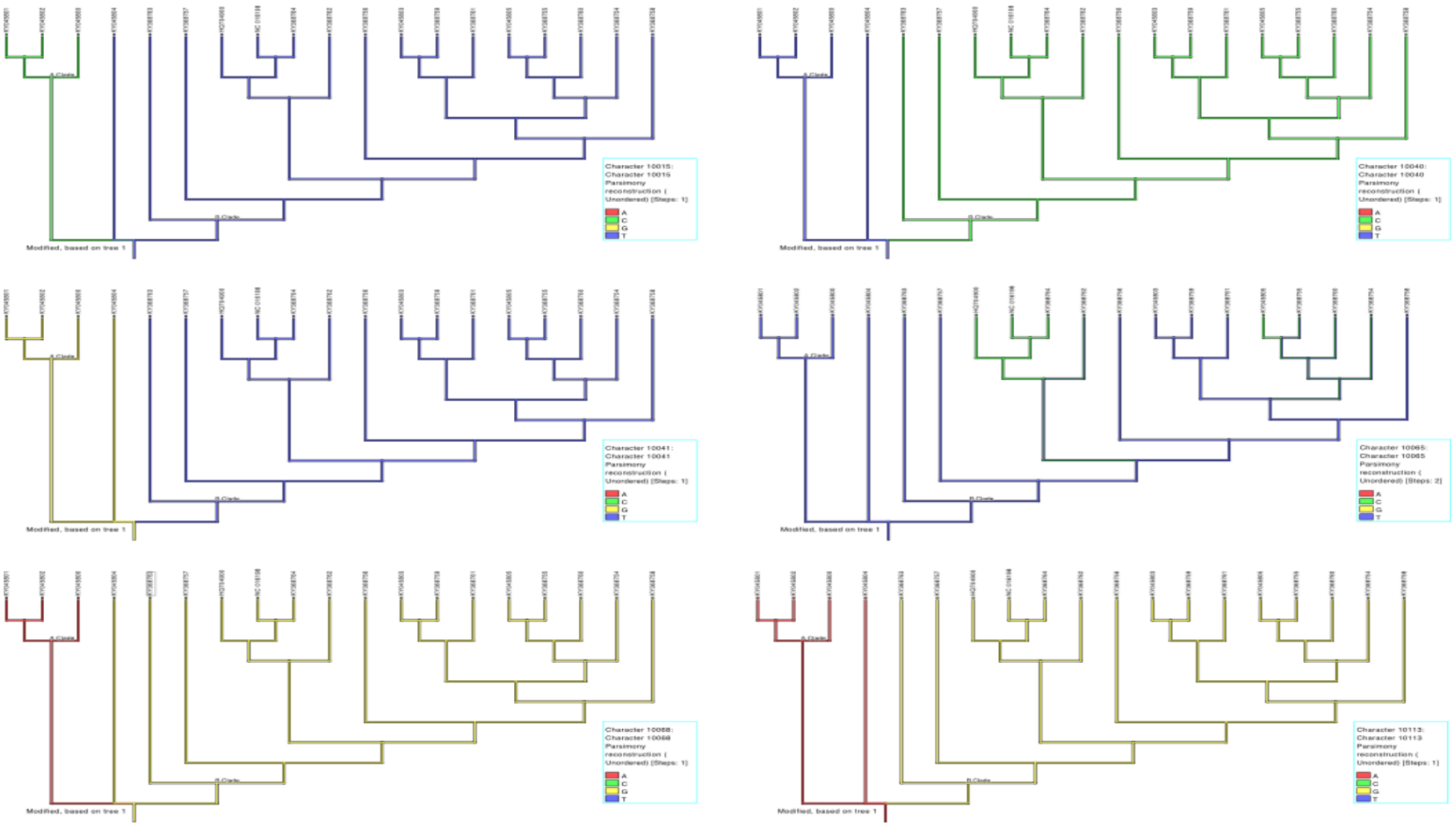


Figure 5.6: 6 trees indicating the 6 SNPs that would be recovered using primers ASC_ND2_F1. The neighbour joining trees are built from the 19 published full mitochondria of Ascaris spp.. The A clade is far left and the B clade is to the right. Each colour represents one of the 4 nucleotides so we can trace how they differentiate both clades and individuals. Most of the SNPs could be used to separate the A and B clades but some can be used to distinguish the tips of the trees.

To begin to assess the degree of variation that could be detected with the primers targeting the grouped SNPs and the COX1 and ITS1 regions, neighbour joining trees were built with modern data. For the COX1 and ITS1 primers, most of the regions amplified produced poorly distinguished clades or none at all (see Figure 5.7 as an example). Any target regions that produced no clades were removed from consideration. Despite the poor power for distinguishing monophyletic groups, the COX1 and ITS1 primers were included in the downstream analysis. Due to the high proportion of the modern literature focused on these gene regions, these targets have the potential to be more informative when comparing between contemporary and ancient *Ascaris* spp. populations.

To assess whether the collection of grouped SNP primers could be used as a proxy for full mitochondrial assessment, a neighbour-joining tree was built using a concatenation of the target regions and compared to a neighbour joining tree built using complete mitochondria. As can be seen in Figure 5.8, the three clades identified by Nejsum et al. (2017) can still be recovered but the tips are not an exact match. These steps were a first assessment of the utility of these primers as we cannot assume that the variation in modern populations encapsulates all of the variation of the past. Finally, for the primers targeting individual SNPs that differentiate the AB clade, a number of primers remained after the quality assessment steps above so 8 were sub-selected to maximise the number of gene regions these primers targeted. For this collection of samples, primers were also indexed so samples could be separated post pooling. The primers were synthesized by Invitrogen and Integrated DNA technologies (IDT) and diluted in house using Qiagen Nuclease Free water to produce a working stock of 10mM. A list of the primers developed can be seen in Supplementary 5.10.2.

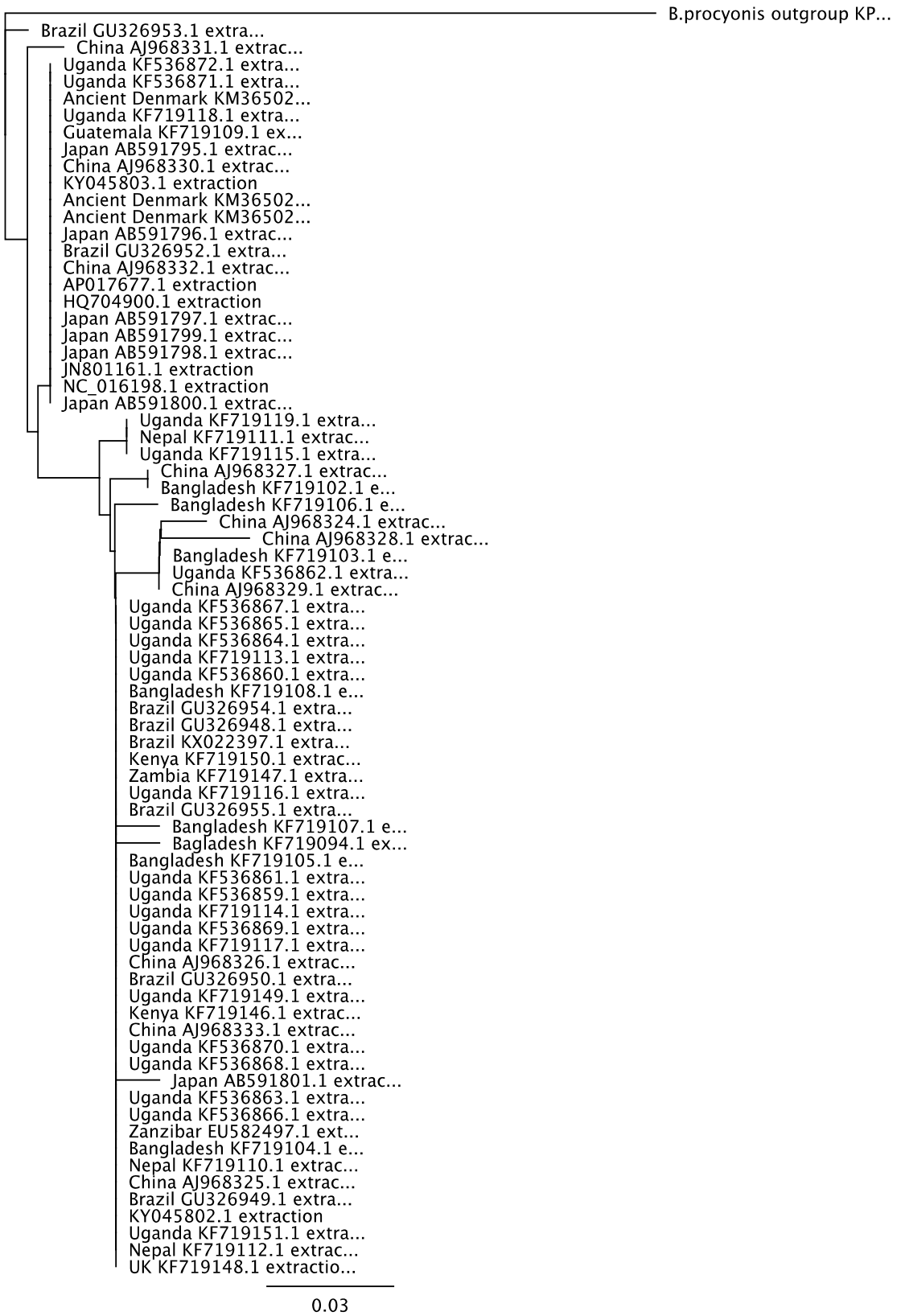


Figure 5.7: A phylogenetic neighbour-joining tree based on 75 published modern COX 1 sequences. The target region of primer Asclum_Cox1_F_1 was extracted from these sequences for the construction of the tree.

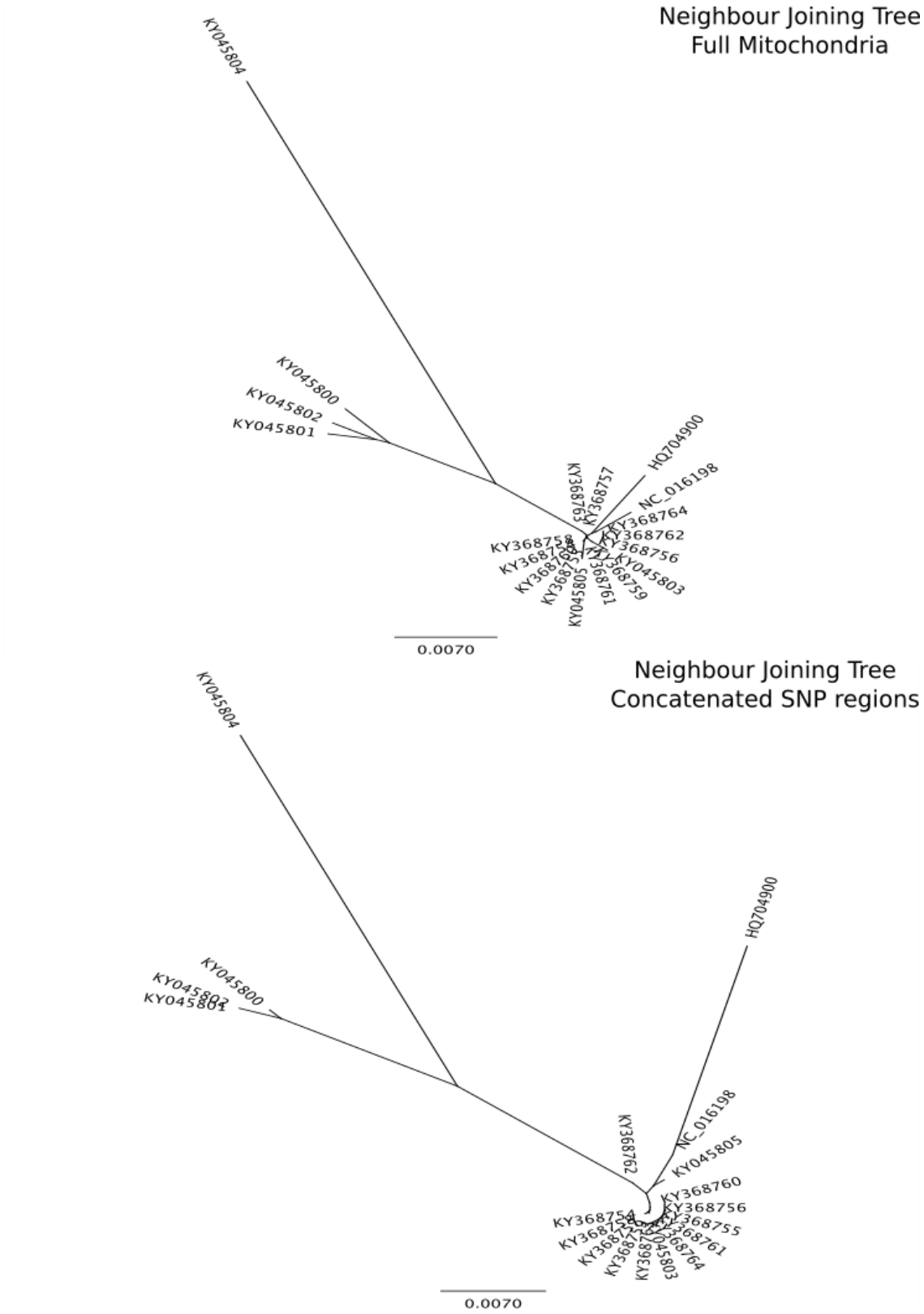


Figure 5.8: Two neighbour joining trees based on the 19 published full mitochondria of Ascaris spp. The top tree was built from the complete genomes whilst the bottom tree was built form a concatenation of the target regions of the primer designed to amplify multiple SNPs. Whilst the main clades of the trees are retained, there are changes to the tips.

5.7.3 Methodology

To maximise the likelihood of these primers working, communal samples that were positive for *Ascaris* spp. eggs by both microscopy and targeted PCR (by Flammer (2014)) were selected, Table 5.5. Communal samples from Odense (ODDK) were also utilised, despite an absence of prior genetic evidence, as extracts from the other sites became low. As the variations in methodology for Tests 1 and 2 made little difference to the results, the original Flammer (2018) protocol was used in Test 3 (as described in the methodology). Targeted PCR was also carried out following the protocol in the methodology, using the new primers developed in this study, along with the *Ascaris lumbricoides* CO1 (241 bp) primer used in Test 2 and *A. lumbricoides* targets developed in Peng (2005) and Wiria et al. (2010). The reads were processed following the protocol in the methodology and reads were aligned to a complete *Ascaris* mitochondria (accession code HQ704900.1).

5.7.4 Results and Discussion

None of the samples produced reads that mapped to the complete *Ascaris* mitochondria, including the primers that had previously worked successfully. For most of the primers, either no reads were recovered or the number of reads were low. See Table 5.6 for the results. This suggests that no endogenous DNA was amplified. The key exception was the pool amplified utilising primer pair ASC_ND1_F1 and ASC_ND1_R1 which produced 32313 reads after data processing. This volume of reads indicated that DNA was amplified, supported by the gel run which showed a band around the right length for our target region (185 bp). This result suggests that the lack of reads recovered with the other primers was not an indicator of inhibitors at play.

The over represented sequences identified by FastQC for the pool amplified by ASC_ND1_F1 were visually assessed to check whether the reads were primer concatemer but this was not the case. The two most common sequence (GCGGCAGGTTGGTGCGCATGAGCAGCCAGACCATCGGCACCGCGAAGACG 28.17% of sequences GCGGCAGGTTGGTGCGCATGAGCAGCCAGACCATCGGCACCGCGAAGACG 18.84% of sequences) from this amplification were run through BLASTn to identify what had been amplified. These sequences predominantly matched up to a range of bacteria but the matches were weak (max E-value 0.037) and there were a number of mismatches. Whilst it cannot be stated with certainty what was amplified, the most probable conclusion is that it was environmental bacterial DNA.

Table 5.5: Samples used for Test 3
Table includes samples, archaeological information, extraction protocol and targets used. Country codes are: NO- Norway, GE- Germany, UK- United Kingdom.

Sample code	Site	Country	Dating	Extraction Type	Targets used
VNOGNS_15195	Goksted Nedre Sandefjord	NO	Viking	Flammer 2018 By PF	Asclum_Cox1_F_A, Asclum_Cox1_F_C, Asclum_Cox1_F_A+D, Asclum_Cox1_F_PENG, Ascaris_Cote_CytB_F, Ascaris_Cote_NAD1_F, Asclum_ITS1_F_D + C, Asclum_ITS1_F_WIRIA, Asclum_COX1_F_1, Asclum_ITS1_F_6, Asclum_ITS1_F_C, ASC_ND1_F1, ASC_CTB_F1, ASC_CTB_F2, ASC_CX2_F1, ASC_ND2_F1, ASC_CX3_F1, Asc_Co1_F2, HR_COX1_1F, HR_COX2_2F, HR_NAD5_3F, HR_ND2_6F, HR_NAD5_4F, HR_NAD1_5F, HR_CYTB_7F, HR_NAD4_8F
VNOGNS_15210	Goksted Nedre Sandefjord	NO	Viking	Flammer 2018 By PF	Asclum_Cox1_F_A, Asclum_Cox1_F_C, Asclum_Cox1_F_A+D, Asclum_Cox1_F_PENG, Ascaris_Cote_CytB_F, Ascaris_Cote_NAD1_F, Asclum_ITS1_F_D + C, Asclum_ITS1_F_WIRIA, Asclum_COX1_F_1, Asclum_ITS1_F_6, Asclum_ITS1_F_C, ASC_ND1_F1, ASC_CTB_F1, ASC_CTB_F2, ASC_CX2_F1, ASC_ND2_F1, ASC_CX3_F1, Asc_Co1_F2, HR_COX1_1F, HR_COX2_2F, HR_NAD5_3F, HR_ND2_6F, HR_NAD5_4F, HR_NAD1_5F, HR_CYTB_7F, HR_NAD4_8F
SDBM_26-278	Bad Mergentheim	GE	c2800 BCE	Flammer 2018 By PF	Asclum_Cox1_F_A, Asclum_Cox1_F_C, Asclum_Cox1_F_A+D, Asclum_Cox1_F_PENG, Ascaris_Cote_CytB_F, Ascaris_Cote_NAD1_F, Asclum_ITS1_F_D + C, Asclum_ITS1_F_WIRIA, Asclum_COX1_F_1, Asclum_ITS1_F_6, Asclum_ITS1_F_C, ASC_ND1_F1, ASC_CTB_F1, ASC_CTB_F2
GBLW_229	London Cesspit	UK	Early Medieval	Flammer 2018 By PF	Asclum_Cox1_F_A, Asclum_Cox1_F_C, Asclum_Cox1_F_A+D, Asclum_Cox1_F_PENG, Ascaris_Cote_CytB_F, Ascaris_Cote_NAD1_F, Asclum_ITS1_F_D + C, Asclum_ITS1_F_WIRIA, Asclum_COX1_F_1, Asclum_ITS1_F_6, Asclum_ITS1_F_C, ASC_ND1_F1, ASC_CTB_F1, ASC_CTB_F2, ASC_CX2_F1, ASC_ND2_F1, ASC_CX3_F1, Asc_Co1_F2, HR_COX1_1F, HR_COX2_2F, HR_NAD5_3F, HR_ND2_6F, HR_NAD5_4F, HR_NAD1_5F, HR_CYTB_7F, HR_NAD4_8F

Sample code	Site	Country	Dating	Extraction Type	Targets used
GBLW_419	London Cesspit	UK	Early Medieval	Flammer 2018 By PF	Asclum_Cox1_F_A, Asclum_Cox1_F_C, Asclum_Cox1_F_A+D, Asclum_Cox1_F_PENG, Ascaris_Cote_CytB_F, Ascaris_Cote_NAD1_F, Asclum_ITS1_F_D + C, Asclum_ITS1_F_WIRIA, Asclum_COX1_F_1, Asclum_ITS1_F_6, Asclum_ITS1_F_C, ASC_ND1_F1, ASC_CTB_F1, ASC_CTB_F2, ASC_CX2_F1, ASC_ND2_F1, ASC_CX3_F1, Asc_Co1_F2, HR_COX1_1F, HR_COX2_2F, HR_NAD5_3F, HR_ND2_6F, HR_NAD5_4F, HR_NAD1_5F, HR_CYTB_7F, HR_NAD4_8F
GBLW_420	London Cesspit	UK	Early Medieval	Flammer 2018 By PF	Asclum_Cox1_F_A, Asclum_Cox1_F_C, Asclum_Cox1_F_A+D, Asclum_Cox1_F_PENG, Ascaris_Cote_CytB_F, Ascaris_Cote_NAD1_F, Asclum_ITS1_F_D + C, Asclum_ITS1_F_WIRIA, Asclum_COX1_F_1, Asclum_ITS1_F_6, Asclum_ITS1_F_C, ASC_ND1_F1, ASC_CTB_F1, ASC_CTB_F2, ASC_CX2_F1, ASC_ND2_F1, ASC_CX3_F1, Asc_Co1_F2, HR_COX1_1F, HR_COX2_2F, HR_NAD5_3F, HR_ND2_6F, HR_NAD5_4F, HR_NAD1_5F, HR_CYTB_7F, HR_NAD4_8F
GBLW_751	London Cesspit	UK	Early Medieval	Flammer 2018 By PF	Asclum_Cox1_F_A, Asclum_Cox1_F_C, Asclum_Cox1_F_A+D, Asclum_Cox1_F_PENG, Ascaris_Cote_CytB_F, Ascaris_Cote_NAD1_F, Asclum_ITS1_F_D + C, Asclum_ITS1_F_WIRIA, Asclum_COX1_F_1, Asclum_ITS1_F_6, Asclum_ITS1_F_C, ASC_ND1_F1, ASC_CTB_F1, ASC_CTB_F2, ASC_CX2_F1, ASC_ND2_F1, ASC_CX3_F1, Asc_Co1_F2, HR_COX1_1F, HR_COX2_2F, HR_NAD5_3F, HR_ND2_6F, HR_NAD5_4F, HR_NAD1_5F, HR_CYTB_7F, HR_NAD4_8F
EOMF_1	East Oxford Minchery farm	UK	Medieval	Flammer 2018 By PF	Asclum_Cox1_F_A, Asclum_Cox1_F_C, Asclum_Cox1_F_A+D, Asclum_Cox1_F_PENG, Ascaris_Cote_CytB_F, Ascaris_Cote_NAD1_F, Asclum_ITS1_F_D + C, Asclum_ITS1_F_WIRIA, Asclum_COX1_F_1, Asclum_ITS1_F_6, Asclum_ITS1_F_C, ASC_ND1_F1, ASC_CTB_F1, ASC_CTB_F2, ASC_CX2_F1, ASC_ND2_F1, ASC_CX3_F1, Asc_Co1_F2, HR_COX1_1F, HR_COX2_2F, HR_NAD5_3F, HR_ND2_6F, HR_NAD5_4F, HR_NAD1_5F, HR_CYTB_7F, HR_NAD4_8F
ODDK_1702	Vilhelm Werners Plads, Odense	DE	Medieval	Flammer 2018 By HR	ASC_ND1_F1, ASC_CTB_F1, ASC_CTB_F2, ASC_CX2_F1, ASC_ND2_F1, ASC_CX3_F1, Asc_Co1_F2

Sample code	Site	Country	Dating	Extraction Type	Targets used
ODDK_2358	Vilhelm Werners Plads, Odense	DE	Medieval	Flammer 2018 By HR	HR_COX1_1F, HR_COX2_2F, HR_NAD5_3F, HR_ND2_6F, HR_NAD5_4F, HR_NAD1_5F, HR_CYTB_7F, HR_NAD4_8F
HLYGV_AS 25 4/7	Gründungsviertel Lübeck	GE	Medieval	Flammer 2018 By PF	ASC_CX2_F1, ASC_ND2_F1, ASC_CX3_F1, Asc_Co1_F2, HR_COX1_1F, HR_COX2_2F, HR_NAD5_3F, HR_ND2_6F, HR_NAD5_4F, HR_NAD1_5F, HR_CYTB_7F, HR_NAD4_8F
HLYGV_AS 25 6/7	Gründungsviertel Lübeck	GE	Medieval	Flammer 2018 By PF	ASC_CX2_F1, ASC_ND2_F1, ASC_CX3_F1, Asc_Co1_F2, HR_COX1_1F, HR_COX2_2F, HR_NAD5_3F, HR_ND2_6F, HR_NAD5_4F, HR_NAD1_5F, HR_CYTB_7F, HR_NAD4_8F

Table 5.6 Results of Test 3
A table summarising the Targeted PCR runs on the primers developed for this study. Any primers that are omitted from this table are excluded as no reads were recovered during the demultiplexing step. Primers that are denoted with the term POOL indicate that the individual extracts were pooled prior to the library build, otherwise the tag number is indicated followed by the primer used.

Primer	Reads	Post trimmomatic	Post usearch	Reads Aligning to *Ascaris* genome
POOL Asclum_Cox1_F_1	206	1	1	0
POOL Asclum_Cox1_FA (RD)	77761	1	1	0
POOL ASC-CTB-F1	33464	21	9	0
POOL ASC-CTB-RF2	190350	1085	113	0
POOL ASC-ND1-F1	2113154	2073737	27505	0
POOL ASC-ND2-F1	114908	1174	177	0
POOL Asc_Co1_f2n_p2	1	1	1	0
2_HR_COX1_1R_N	1	0	0	0
4_HR_COX1_1R_N	1	0	0	0
5_HR_COX1_1R_N	1	0	0	0
6_HR_COX1_1R_N	2	1	1	0
13_HR_COX1_1R_N	1	0	0	0
25_HR_COX2_2F_N	1	1	1	0
28_HR_COX2_2F_N	4	3	3	0
44_HR_NAD5_3R_N	1	0	0	0
45_HR_NAD5_3R_N	1	0	0	0
60_HR_NAD5_4F_N	7	3	3	0
61_HR_NAD5_4F_N	4	0	0	0
62_HR_NAD5_4F_N	3	1	1	0
63_HR_NAD5_4F_N	1	1	1	0
67_HR_NAD5_4F_N	4	1	1	0
68_HR_NAD5_4F_N	1	0	0	0
69_HR_NAD5_4F_N	6	2	2	0
70_HR_NAD5_4F_N	5	1	1	0
71_HR_NAD5_4F_N	1	0	0	0
80_HR_NAD1_5F_N	33	9	9	0
81_HR_NAD1_5F_N	36	11	11	0
82_HR_NAD1_5F_N	84	23	23	0
84_HR_NAD1_5F_N	37	10	10	0
85_HR_NAD1_5F_N	3	0	0	0
87_HR_NAD1_5F_N	34	7	7	0
90_HR_NAD1_5F_N	49	13	13	0
91_HR_NAD1_5F_N	319	79	79	0
92_HR_NAD1_5F_N	24	4	4	0
93_HR_NAD1_5F_N	4	2	2	0
101_HR_ND2_6R_N	5	4	4	0
102_HR_ND2_6R_N	9	8	8	0
103_HR_ND2_6R_N	1	1	1	0
104_HR_ND2_6R_N	1	1	1	0
105_HR_ND2_6R_N	4	2	2	0
106_HR_ND2_6R_N	3	3	3	0
110_HR_ND2_6R_N	2	0	0	0
121_HR_CYTB_7F_N	1	1	1	0
122_HR_CYTB_7F_N	1	0	0	0
123_HR_CYTB_7F_N	1	0	0	0
125_HR_CYTB_7F_N	1	1	1	0

132_HR_CYTB_7F_N	3	3	3	0
145_HR_NAD4_8R_N	1	0	0	0
146_HR_NAD4_8R_N	1	1	1	0
147_HR_NAD4_8R_N	3	0	0	0

5.8 Conclusion

There were two aspects of genetic methodology that needed to be explored before a large-scale analysis of genetic variance in *Ascaris* spp. could be undertaken: is shotgunning better than targeted PCR for detecting helminths in archaeological contexts and can either of these sequencing approaches produce the data required for modelling population dynamics? This study has established that a shotgunning approach can be successfully applied to archaeological sediment to assess the helminth community; in communal samples, coprolites and individual burials. Whilst a wide range of helminth species were identified, the human gastrointestinal taxa were identified by the highest number of robust hits. The advantage of the shotgunning methodology used here is that it can be applied to small amounts of starting material, and therefore it is suitable for assessing changes to the helminth community between individual burials or between strata within a communal deposit. Whilst assessing change to the helminth composition through strata provides an overview of changes to a population over time, individual burial analysis provides us with the capacity to link helminth genetic data to an individual host and time point.

In addition to this success, this study has also established that the shotgunning approach was not as sensitive as targeted PCR, as the latter identified a higher number of positive samples when a single helminth species was being traced. This suggests that the helminths identified by shotgunning in this study are a subset of the complete picture. This issue is exacerbated by the uncertainty linked to the incomplete nature of the helminth reference sequences. As well as incomplete, the identification of helminth taxa that seemed out of place considering the context of the samples (*P. trichosuri, E. elaphi*), indicates that matches should be considered critically as contamination is a problem for the helminth database. A third option that has not been explored in this chapter is capture via hybridization. This provides greater enrichment for endogenous DNA than shotgunning but also allows a wider number of genetic regions and species to be targeted than amplicon PCR. It is possible that a combination of deeper sequencing, enrichment and a higher number of complete helminth reference genomes will change this result but for now, a targeted approach is the most appropriate tool for a wide scale analysis of single graves.

The primers developed for this study targeting variable *Ascaris* spp. regions produced no endogenous reads despite the success of the targeted approach using other primers earlier in this study. The low number of published studies using genetic techniques in palaeoparasitology suggests that the problems detected in this study are not unique. A number of factors may influence the reliability of this approach. The first is environmental inhibitors such as fulmic acids, humic acids, metal ions and polyphenol which can absorb or cause crosslinking of nucleic acids and potentially reduce the specificity of primers.

Another issue when applying genetics to palaeoparasitology is the very low quantity of endogenous, degraded helminth DNA in a mixed context. In this study, primers developed to target the ND1 gene of *Ascaris* spp. instead amplified bacterial genomes despite the thorough pre-processing steps taken during the primer design. This result suggests that even if the primers designed are specific, the high ratio of exogenous to endogenous DNA combined with inhibitors reducing the specificity of primers will sometimes result in spurious, or no PCR product. Whilst these issues outlined above will be present in all types of palaeoparasitological study, they are likely to be more problematic when working with single graves in comparison to communal contexts. Before a widescale analysis of *Ascaris* spp. from single graves in the UK can be undertaken, alternative primers which can be more reliably applied need to be developed.

This study has established that a targeted PCR approach identified more positive samples for *Ascaris* spp. than shotgunning however, currently the primers that have been published for *Ascaris* spp. are not sufficient for assessing population dynamics and routes of infection. Whilst short variable targets have been designed for analysis of ancient *T. trichiura* (Flammer et al., 2018), this is the first study to try and emulate this approach with *Ascaris* spp.. Both of these helminths are very common but there are cases where one is far more abundant than the other, as was found in Chapter 3. If variable *Ascaris* spp. targets can be developed successfully in future studies, the ability to assess population dynamics of two soil transmitted helminth will strengthen the interpretation of these organisms and how their population dynamics can be related to their human hosts. The approach taken here shifts the focus of research from species identification to maximising the information that can be recovered from the most common helminths found in archaeological contexts, and this focus will only grow in future research.

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5.10 Supplementary

5.10.1 Detailed Methodology

Extraction Buffers

Haile (2012) Sediment extraction protocol

PB buffer, AE elution buffer (Qiagen) and Salton wash 1 and 2 buffer (BIO 101).
Made in house: Bulat extraction buffer
0.02 g/mL Sarcosyl, 50 mM Tris–HCl (pH 8.0), 20 mM NaCl, 3.5% 2-mercaptoethanol, 50 mM 1,4-Dithio-L-threitol (DTT), 2 mM N-phenacylthiazone bromide (PTB), 0.8 g/mL Proteinase K

Dabney (2013) extraction protocol

PE buffer (Qiagen)

Made in house: TET elution buffer

Reagent	Final concentration	Volume (50mL)	Volume (1L)
0.5 M EDTA, pH 8.0	1 mM	100 µl	2000 µl
1 M Tris-HCl, pH 8.0	10 mM	500 µl	10 mL
Tween 20	0.05%	25 µl	500 µl
Water		~49.4 ml	~988 mL

Made in house: Binding buffer

Reagent	Final concentration	Volume/amount (50mL	Volume/amount (1L)
Guanidine hydrochloride	5 M	23.88g	477.60g
Water		to 30 mL	to 600 mL
Isopropanol	40%	to 50 mL	to 1L
Tween 20	0.05%	25 µL	500 µL

Library build protocols
Targeted Approach
Targeted PCR
The targeted PCR steps were based on those outlined by Flammer et al. (2018). Two rounds of PCR were undertaken: the first round uses pure template DNA whilst the second round used 5 µl of the first round PCR solution as the template. Two separate PCR solutions therefore were made, the latter replacing the template DNA portion with water to keep the buffer concentrations at the right ratio. The PCR solution consists of (per sample):
AmpliTaq: 0.20 µl
10x Buffer: 2.50 µl
$MgCl_2$: 2.00 µl
Primers (forward and reverse): 2X 0.50 µl
Template DNA/H_2O: 1.00 µl
dNTPs: 1.00 µl
H_2O: 17.30 µl (to make up to 25 µl)

The library builds consisted of 40 cycles per round, with the Tm of the primers dictating the annealing temperature:
PCR Run
95°C- 5 minutes
Cycle Steps
95°C- 20 seconds
Tm based – 20 seconds – 1 minutes
72°C – 20 seconds
40x Cycles
72°C – 7 minutes

Gel electrophoresis
A 1.5% gel was used by combining 3g of agarose in 200 ml of 1X borate buffer. 2 μl of stock GelRed was added before the buffer was microwaved for 5 minutes. Once the gels had cooled and were loaded they were run at 120V for 40-45 minutes.

Shotgun approach
Illumina Blunt End Library Build
This Adaptor Ligation approach was based on the methodology in Meyer and Kircher (2010). This consists of multiple steps, the first being blunt end repair and the master mix consisted of (volume per sample):
H_2O: 17.12 μl
Buffer Tango (10X): 7.00 μl
dNTPs (25 mM each): 0.28 μl
ATP (100mM): 0.70 μl
T4 polynucleotide kinase: 3.50 μl
T4 DNA polymerase: 1.40 μl

30 μl of master mix was then combined with 40 μl of sample before incubating in a thermal cycler for 15 minutes at 25 °C followed by 5 minutes at 12 °C. The mix was then purified using a Qiagen MinElute silican spin column to bind, wash and then elute the DNA. The next step was adaptor ligation and the master mix consisted of (volume per sample):
H_2O: 10.50 μl
T4 DNA ligase buffer (10X): 4.00 μl
PEG-4000 (50%): 4.00 μl
Adaptor mix (100 mM each): 0.50 μl
T4 DNA ligase: 1.00 μl

20 μl of master mix was then added to the eluted DNA and incubated for 30 minutes at 22 °C in a thermal cycler. Again the mixture was purified using spin columns (Qiaquick columns) by employing the bind, wash and eluting steps. The final step was to build a complimentary sequence to the single stranded adaptor. The master mix was (volume per sample):
H_2O: 14.10 μl
Thermopol reaction buffer (10X): 4.00 μl
dNTPs (25mM each): 0.40 μl
Bst polymerase, large fragment: 1.50 μl

20 μl of the master mix was then added to the eluted DNA, mixed well and incubated in a thermal cycler for 20 min at 37°C. To inactivate the enzyme, the samples were then left to freeze over-night.

PCR of libraries
After the adaptor ligation, QPCR was undertaken to roughly quantify the amount of endogenous DNA that was present in the samples. The QPCR master mix consisted of (per sample):
BSA 20 mg/ml: 2.00 μl
IS4 (2mM): 2.00 μl
Tag_51 (2mM): 2.00 μl
labTAQ: 10.00 μl
H_2O: 3.00 μl
Library: 1.00 μl

The samples were run through Thermo-Fisher StepOne Real-Time PCR System for QPCR and the results were used to assess which samples should be further processed and the optimum number of cycles for the PCR amplification. The PCR solution consisted of (per sample):
BSA 10 mg/ml: 4.00 μl
IS4 (2mM): 3.00 μl
Bst Polymerase: 25.00 μl
Index: 3.00 μl
Library: 15.00 μl

PCR Run
95°C- 2 minutes
Cycle Steps
95°C- 15 seconds
60°C – 30 seconds
68°C – 30 seconds
Various Cycles
68°C – 7 minutes

Data processing
Commands
Trimming for illumina primers and removing sequences below 36 bp for MiSeq runs.

```
java -jar trimmomatic-0.38.jar PE -phred33 File_1.fastq
File_2.fastq File_1Ptrim.fq File_1UPtrim.fq File_2Ptrim.fq
File_2UPtrim.fq ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10 LEADING:30
TRAILING:30 MINLEN:36
```

Trimming for illumina primers and removing sequences below 25 bp for HiSeq runs.

```
java -jar trimmomatic-0.38.jar SE -phred33 Fileinput.fq.gz
File_trimHR.fq ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10 LEADING:30
TRAILING:30 SLIDINGWINDOW:4:30 MINLEN:25
```

#Dereplication

```
./usearch -derep_fulllength File_trimHR.fq -fastqout
File_trimHR_derep.fq -sizeout
```

#Removal of PolyA tails

```
./UrQt --in HR15_trimHR2_derep.fq –out
HR15_trimHR2_derep_PolyA.fastq --N A
```

#BWA Alignment

```
bwa index -p Title_refseq referenceseq.fasta
bwa mem Title_refseq File_trimHR_derep_otus.fq >
File_trimHR_derep_otus.sam
samtools view -b -S File_trimHR_derep_otus.sam >
File_trimHR_derep_otus.bam
samtools sort File_trimHR_derep_otus.bam >
File_trimHR_derep_otus _sort.bam
samtools index File_trimHR_derep_otus_sort.bam
samtools flagstat
```

#BLASTn Run

```
blastdb_aliastool -dblist "Para_ref_seq3 /media/jbod/raid5-
sdb/blastdb/nt" -dbtype nucl -out Para+ncbi19 -title "Parasit
and NCBI alias 2019"
./fastq_to_fasta -Q33 -i -o File_trimHR_derep_otus.fq
blastn -query File_trimHR_derep_otus.fa  -db Para+ncbi19 -out
File_trimHR_derep_otus.xml -num_threads 10 -outfmt 5
```

5.10.2 Primers designed and used

Targets designed prior to this study and used in the Shotgun versus Targeted PCR Test (Test 2).

Trichuris Trichiura ITS1

Primer Name	Sequence	Product Length	Designed by
Tri_ITS1_3F	GCTCGATCAGGCAGCAGCGG	459BP	Flammer 2018
Tri_ITS1_3F_N	GCTCGATCAGGCAGCAGCGGTT		
Tri_ITS1_4R	AATTCACGTCAATTCTCGAG		
Tri_ITS1_4Rn	AATTCACGTCAATTCTCGAGTG		

Cryptosporidium spp. 18S

Primer Name	Sequence	Product Length	Designed by
Cryptosporidium_18S_F	GAACTGTACAGATGCTTGGGAGAAT	Variable	Flammer
Cryptosporidium_18S_R	CCTTCGTTAGTTGAATCCTCTTTCCA		

Taenia solium COX1

Primer Name	Sequence	Product Length	Designed by
T.solium_COX1_F1 CO1	TTTATACTTTGTTGGGTTTGTG	202BP	Flammer
T.solium_COX1_R1 CO1	AATGGAATTAAGTATTTACCA		

Taenia saginata COX1

Primer Name	Sequence	Product Length	Designed by
T.saginata_COX1_R2 CO1	TAGACGCACCCGCCAAATGCAACG	199BP	Flammer
T.saginata_COX1_F2 CO1	TTATCTGATTTGAATTTACCCCG		

16S Universal Primer

Primer Name	Sequence	Product Length	Designed by
UNI_16S F	ATAACACGAGAAGACCCT	Variable	Horreo et al. 2013
UNI_16S R	CCCGCGGTCGCCCCAAC		

Ascaris sp. CO1. This primer was also applied during Tests 2 and 3.

Primer Name	Sequence	Product Length	Designed by
Asc_CO1-F2	ACTCTCGGGCTTATTTTACTGC	241BP	Flammer 2018
Asc_CO1-R	ACATAATGAAAATGACTAACAAC		
Asc_CO1-F2n_p2	CTCTCGGGCTTATTTTACTGCTG		
Asc_CO1-Rn_p2	ACATAATGAA AATGACTAACAACTA		

Targets developed in this study: *Ascaris sp.* COX1 and ITS1 targets

Primer Name	Sequence	Product Length	Designed by
Asclum_Cox1_F_A	TTGGTATTATTAGTCAGAGTAGTT	80 BP	This study
Asclum_Cox1_R_A	AAATAGCATAAACCATACCC		
Asclum_Cox1_F_C	GGGTATGGTTTATGCTATTT	77 BP	This study
Asclum_Cox1_R_C	ATACCAACAGTATACATATGATG		
Asclum_Cox1_F_A	TTGGTATTATTAGTCAGAGTAGTT	74 BP	This study
Asclum_Cox1_R_D	ATAAACCATACCCAAAGAC		
Asclum_Cox1_F_1	TGGTTGGCTACCTTGTTTGGT	100 BP	
Asclum_Cox1_R_1	CCCCGGTTAACCCACCAATAG		
Asclum_Cox1_F_PENG	TTTTTTGGTCATCCTGAGGTTTAT	384-450 BP	Peng 2005
Asclum_Cox1_R_PENG	GCATCACAATAGCCAACAAATAC		
Asclum_ITS1_F_C	TGATGTAATAGCAGTCGGCG	182 BP	This study
Asclum_ITS1_R_C	TAGCTCGGTGAAGCGTAGA		
Asclum_ITS1_F_D	GTAATAGCAGTCGGCGGTTTCTT	178 BP	This study
Asclum_ITS1_R_C	TAGCTCGGTGAAGCGTAGA		
Asclum_ITS1_F_6	AATAGGTGGCATGTTGGGCT	113BP	This study
Asclum_ITS1_R_6	CAGGCGATAGCTCGGTGAAG		
Asclum_ITS1_F_WIRIA	GTAATAGCAGTCGGCGGTTTCTT	87BP	Wiria et al. 2010
Asclum_ITS1_R_WIRIA	GCCCAACATGCCACCTATTC		

Targets developed in this study: *Ascaris sp.* Multiple SNPs within 100 BP (SNPs identified by Nejsum et al. (2017))

Primer Name	Sequence	Product Length	Designed by

ASC_CX2_F1	TGTAGGCTTTTGTTTGGTGTTT	243BP	This study
ASC_CX2_R1	CTTCAATACCACTGATGCCCA		
ASC_ND1_F1	AGTTGTTACCGGTGTCTTTGA	185BP	This study
ASC_ND1_R1	AAACCACCACCAAAAGTCCT		
ASC_ND2_F1	TGTTTTGGTTTGATGAAGTGTGT	188BP	This study
ASC_ND2_R1	GAGCCACACCAACCTTCATC		
ASC_CTB_F1	TCTAATGATGGTACTTTGGCCTT	176BP	This study
ASC_CTB_R1	ACCCAAACCTTCTTCAGACGA		
ASC_CTB_F2	GGTTTTGGGCTTCTGTGGTTA	203 BP	This study
ASC_CTB_R2	AGTTCTTCCAGTCTCATGCAAAA		
ASC_CX3_F1	TGGATGGTTTTAAGTTTGGTGTT	122 BP	This study
ASC_CX3_R1	CCAGACACCACCAACATCATG		

Targets developed in this study: *Ascaris sp.* Individual AB SNPs (SNPs identified by Nejsum et al. (2017))

Primer Name	**Sequence**	**Product Length**	**Designed by**
HR_COX1_1F	TTCTTTTGGGTAGTGGTCAGTT	100BP	This study
HR_COX1_1F_N	TTCTTTTGGGTAGTGGTCAGTTAT		
HR_COX1_1R	CCAAAACCACCAATCATAGTAGG		
HR_COX1_1R_N	CCAAAACCACCAATCATAGTAGGTA		
HR_COX2_2F	AGTTTTCCTGTGGTTGGTGT	90BP	This study
HR_COX2_2F_N	AGTTTTCCTGTGGTTGGTGTAT		
HR_COX2_2R	TCCAAAAGAGTAACTTCCAAAGCCAC		
HR_COX2_2R_N	TCCAAAAGAGTAACTTCCAAAGCCACAGG		
HR_NAD5_3F	AGTTAGTTGGGATTTGTTGGGT	79BP	This study
HR_NAD5_3F_N	AGTTAGTTGGGATTTGTTGGGTAT		
HR_NAD5_3R	AGCCCCTCTACACCTATCTCA		
HR_NAD5_3R_N	AGCCCCTCTACACCTATCTCAAT		
HR_NAD5_4F	TGCTAAAGGTTGGGTTTATGGA	78BP	This study
HR_NAD5_4F_N	TGCTAAAGGTTGGGTTTATGGATTG		
HR_NAD5_4R	TACACCAAGAGAATTAACACCCC		

HR_NAD5_4R_N	GTTACACCAAGAGAATTAACACCCC		
HR_NAD1_5F	TGGTGTTTGTGCGTAGTTCT	87BP	This study
HR_NAD1_5F_N	TGGTGTTTGTGCGTAGTTCTTA		
HR_NAD1_5R	TCAAAGACACCGGTAACAACT		
HR_NAD1_5R_N	TCAAAGACACCGGTAACAACTTA		
HR_ND2_6F	TGTTTTGGTTTGATGAAGTGTGT	82BP	This study
HR_ND2_6F_N	TGTTTTGGTTTGATGAAGTGTGTTT		
HR_ND2_6R	ACCAGCATAAGAACCAGACCC		
HR_ND2_6R_N	ACCAGCATAAGAACCAGACCCCT		
HR_CYTB_7F	TGGGTTTACGGTTTCTAGTGCT	86BP	This study
HR_CYTB_7F_N	TGGGTTTACGGTTTCTAGTGCTAC		
HR_CYTB_7R	ACAAAACTAACAACAACAACCCC		
HR_CYTB_7R_N	ACAAAACTAACAACAACAACCCCCA		
HR_NAD4_8F	TATGGTTACCTAAGGCTCATGTAG	88BP	This study
HR_NAD4_8F_N	ATGGTTACCTAAGGCTCATGTAGAG		
HR_NAD4_8R	AAAATCCAGCAGTCCCCAAC		
HR_NAD4_8R_N	AAAATCCAGCAGTCCCCAACTT		

Targets developed in this study: *Ascaris sp.* Individual AB SNPs (SNPs identified by Nejsum et al. (2017))- Pooling Tags

Primer Name	**Sequence**
002_HR_COX1_1R_N	TTCTCCACCAAAACCACCAATCATAGTAGGTA
004_HR_COX1_1R_N	AACACCTCCAAAACCACCAATCATAGTAGGTA
005_HR_COX1_1R_N	CTCTCACCCAAAACCACCAATCATAGTAGGTA
006_HR_COX1_1R_N	TGCGCAACCAAAACCACCAATCATAGTAGGTA
007_HR_COX1_1R_N	GACACAGCCAAAACCACCAATCATAGTAGGTA
008_HR_COX1_1R_N	ACCCCATCCAAAACCACCAATCATAGTAGGTA
010_HR_COX1_1R_N	TACACGACCAAAACCACCAATCATAGTAGGTA
012_HR_COX1_1R_N	ATCTCGTCCAAAACCACCAATCATAGTAGGTA
013_HR_COX1_1R_N	CACACTCCCAAAACCACCAATCATAGTAGGTA
015_HR_COX1_1R_N	GTCTCTGCCAAAACCACCAATCATAGTAGGTA
020_HR_COX2_2F_N	CACCACTAGTTTTCCTGTGGTTGGTGTAT

021_HR_COX2_2F_N	TTCGAACAGTTTTCCTGTGGTTGGTGTAT
023_HR_COX2_2F_N	AACCAAGAGTTTTCCTGTGGTTGGTGTAT
024_HR_COX2_2F_N	CCCTAATAGTTTTCCTGTGGTTGGTGTAT
025_HR_COX2_2F_N	TGCAAGCAGTTTTCCTGTGGTTGGTGTAT
026_HR_COX2_2F_N	GACCAGAAGTTTTCCTGTGGTTGGTGTAT
027_HR_COX2_2F_N	ACCTAGGAGTTTTCCTGTGGTTGGTGTAT
028_HR_COX2_2F_N	CTCGAGTAGTTTTCCTGTGGTTGGTGTAT
029_HR_COX2_2F_N	TACCATCAGTTTTCCTGTGGTTGGTGTAT
030_HR_COX2_2F_N	GCCTATAAGTTTTCCTGTGGTTGGTGTAT
040_HR_NAD5_3R_N	TCCGGATAGCCCCTCTACACCTATCTCAAT
042_HR_NAD5_3R_N	AACTGGAAGCCCCTCTACACCTATCTCAAT
044_HR_NAD5_3R_N	TTCAGGTAGCCCCTCTACACCTATCTCAAT
045_HR_NAD5_3R_N	GACTGTCAGCCCCTCTACACCTATCTCAAT
046_HR_NAD5_3R_N	ACCGGTAAGCCCCTCTACACCTATCTCAAT
047_HR_NAD5_3R_N	CTCAGTGAGCCCCTCTACACCTATCTCAAT
048_HR_NAD5_3R_N	TGCCGTTAGCCCCTCTACACCTATCTCAAT
049_HR_NAD5_3R_N	ACCATCCAGCCCCTCTACACCTATCTCAAT
050_HR_NAD5_3R_N	CTCCTCAAGCCCCTCTACACCTATCTCAAT
051_HR_NAD5_3R_N	TGCTTCGAGCCCCTCTACACCTATCTCAAT
060_HR_NAD5_4F_N	GTCCTGTTGCTAAAGGTTGGGTTTATGGATTG
061_HR_NAD5_4F_N	AACGTTCTGCTAAAGGTTGGGTTTATGGATTG
062_HR_NAD5_4F_N	CCCATTATGCTAAAGGTTGGGTTTATGGATTG
063_HR_NAD5_4F_N	TTCCTTGTGCTAAAGGTTGGGTTTATGGATTG
066_HR_NAD5_4F_N	GGATCCATGCTAAAGGTTGGGTTTATGGATTG
067_HR_NAD5_4F_N	AAAGCCGTGCTAAAGGTTGGGTTTATGGATTG
068_HR_NAD5_4F_N	CCAACCTTGCTAAAGGTTGGGTTTATGGATTG
069_HR_NAD5_4F_N	TGATCACTGCTAAAGGTTGGGTTTATGGATTG
070_HR_NAD5_4F_N	GAAGCAATGCTAAAGGTTGGGTTTATGGATTG
071_HR_NAD5_4F_N	ACAACAGTGCTAAAGGTTGGGTTTATGGATTG
080_HR_NAD1_5F_N	CAAGCTTTGGTGTTTGTGCGTAGTTCTTA

081_HR_NAD1_5F_N	GTATACCTGGTGTTTGTGCGTAGTTCTTA
082_HR_NAD1_5F_N	AGAGACATGGTGTTTGTGCGTAGTTCTTA
084_HR_NAD1_5F_N	TCACACTTGGTGTTTGTGCGTAGTTCTTA
085_HR_NAD1_5F_N	GGAGAACTGGTGTTTGTGCGTAGTTCTTA
087_HR_NAD1_5F_N	CCACAAGTGGTGTTTGTGCGTAGTTCTTA
090_HR_NAD1_5F_N	ACACAGATGGTGTTTGTGCGTAGTTCTTA
091_HR_NAD1_5F_N	CTATAGGTGGTGTTTGTGCGTAGTTCTTA
092_HR_NAD1_5F_N	TGAGAGTTGGTGTTTGTGCGTAGTTCTTA
093_HR_NAD1_5F_N	GCACATCTGGTGTTTGTGCGTAGTTCTTA
100_HR_ND2_6R_N	GCATGCTACCAGCATAAGAACCAGACCCCT
101_HR_ND2_6R_N	AGAAGACACCAGCATAAGAACCAGACCCCT
102_HR_ND2_6R_N	CAACGAAACCAGCATAAGAACCAGACCCCT
103_HR_ND2_6R_N	TCATGAGACCAGCATAAGAACCAGACCCCT
104_HR_ND2_6R_N	GTAGGATACCAGCATAAGAACCAGACCCCT
105_HR_ND2_6R_N	AAACGGCACCAGCATAAGAACCAGACCCCT
106_HR_ND2_6R_N	CCATGGAACCAGCATAAGAACCAGACCCCT
108_HR_ND2_6R_N	GGAAGGTACCAGCATAAGAACCAGACCCCT
109_HR_ND2_6R_N	ACATGTCACCAGCATAAGAACCAGACCCCT
110_HR_ND2_6R_N	CTAGGTAACCAGCATAAGAACCAGACCCCT
121_HR_CYTB_7F_N	CAATTGCTGGGTTTACGGTTTCTAGTGCTAC
122_HR_CYTB_7F_N	TCAGTGATGGGTTTACGGTTTCTAGTGCTAC
123_HR_CYTB_7F_N	GTAATGGTGGGTTTACGGTTTCTAGTGCTAC
124_HR_CYTB_7F_N	AGACTGTTGGGTTTACGGTTTCTAGTGCTAC
125_HR_CYTB_7F_N	CCAGTTCTGGGTTTACGGTTTCTAGTGCTAC
127_HR_CYTB_7F_N	GGACTTGTGGGTTTACGGTTTCTAGTGCTAC
130_HR_CYTB_7F_N	AAGTCCATGGGTTTACGGTTTCTAGTGCTAC
132_HR_CYTB_7F_N	TTGACCTTGGGTTTACGGTTTCTAGTGCTAC
133_HR_CYTB_7F_N	GAGTCACTGGGTTTACGGTTTCTAGTGCTAC
134_HR_CYTB_7F_N	ACGGCAATGGGTTTACGGTTTCTAGTGCTAC
140_HR_NAD4_8R_N	TAGTCGTAAAATCCAGCAGTCCCCAACTT

141_HR_NAD4_8R_N	GTGACTCAAAATCCAGCAGTCCCCAACTT
142_HR_NAD4_8R_N	AGGCCTAAAAATCCAGCAGTCCCCAACTT
143_HR_NAD4_8R_N	CAGTCTGAAAATCCAGCAGTCCCCAACTT
144_HR_NAD4_8R_N	TCGGCTTAAAATCCAGCAGTCCCCAACTT
145_HR_NAD4_8R_N	AGGTACCAAAATCCAGCAGTCCCCAACTT
146_HR_NAD4_8R_N	CAGGACAAAAATCCAGCAGTCCCCAACTT
147_HR_NAD4_8R_N	TCGAACGAAAATCCAGCAGTCCCCAACTT
148_HR_NAD4_8R_N	GTGCACTAAAATCCAGCAGTCCCCAACTT
149_HR_NAD4_8R_N	AAGGAACAAAATCCAGCAGTCCCCAACTT

5.10.3 Lab work completed but excluded from the main body of the chapter

Testing different extraction protocols

Considering the mixed results and the inconsistency of the shotgunning sequencing in the Test 1, it was decided that the communal soil samples SDBM 26-728, SDBM 27-329, ZPO 6625, ZPO 12156, HLGV AS27 4/5, HLGV FS26 1/3, BFR 1036-1156, BFR 2038-2567, Hg, VDKVS K301 and, VDKVS K304 should be re-run based on the sediment protocol developed by Haile (2012) rather than the protocol developed by Flammer (2018). As well as testing whether the protocol improved the shotgunning results, these samples were not sieved during the extraction process to assess whether there was a loss of eggs during sieving. These samples were extracted, amplified, sent for shotgun sequencing and the data was processed following the protocols laid out in the methodology. The number of BLAST matches were significantly lower than for the preliminary run so the remaining extract was utilised for a second library build, sequencing and data analysis and it was only through a process of elimination that it was recognised that the final incubation step had been omitted, hence the poor results. The runs are summarised below.

Sample code	Site	Country	Dating	Sample Type	Extraction type
ZPO 6625	Zurich Opéra	CH	c3060 BCE	Unfiltered soil samples	Phenol-Chloroform and Bulat Buffer
ZPO 12156	Zurich Opéra	CH	c3630 BCE	Unfiltered soil samples	Phenol-Chloroform and Bulat Buffer
SDBM 26-728	Bad Mergentheim	GE	c2800 BCE	Unfiltered soil samples	Phenol-Chloroform and Bulat Buffer
SDBM 27-329	Bad Mergentheim	GE	c2800 BCE	Unfiltered soil samples	Phenol-Chloroform and Bulat Buffer
HLGV AS27 4/5	Gründerviertel Lübeck	GE	c1300 CE	Unfiltered soil samples	Phenol-Chloroform and Bulat Buffer
HLGV FS26 1/3	Gründerviertel Lübeck	GE	c1625 CE	Unfiltered soil samples	Phenol-Chloroform and Bulat Buffer
BFR 1036-1156	Finzel's Reach Bristol	UK	c1150 CE	Unfiltered soil samples	Phenol-Chloroform and Bulat Buffer
BFR 2038-2567	Finzel's Reach Bristol	UK	c1500 CE	Unfiltered soil samples	Phenol-Chloroform and Bulat Buffer
Hg	Hungate York	UK	c900 CE	Unfiltered soil samples	Phenol and Bulat Buffer -test extraction
Hg	Hungate York	UK	c900 CE	Unfiltered soil samples	Chloroform and Bulat Buffer -test extraction
VDKVS K301	Viborg Søndersø	DK	c1030 CE	Unfiltered soil samples	Phenol-Chloroform and Bulat Buffer
VDKVS K304	Viborg Søndersø	DK	c1030 CE	Unfiltered soil samples	Phenol-Chloroform and Bulat Buffer
Blank	Blank	Blank	Blank	Unfiltered soil samples	Phenol-Chloroform and Bulat Buffer

Flotation Pilot Test

Methods

An alternative technique to sieving would be to use flotation. This is a technique regularly and successfully used in modern parasitology studies (see Kilani & Sekla 1987; Marquardt 1961; Satchwell 1986). The gravity of our parasites of interest was established by two past studies: David and Lindquist (1982) and Sawitz (1942). Based on these papers it was decided that the specific gravity of the flotation solution had to be 1.3 or greater. A range of floatation solutions were selected based on published research to test the efficiency of each. Some of these solutions are more likely to result in osmotic shock, destroying the eggs, so this was also considered in the selection of the solutions. We tested: saturated salt, saturated sugar, saturated Magnesium Sulphate, Glycerol and Percoll. The latter, despite having a lower density, was included as it has low osmolarity and could be utilised to remove lighter material such as bacteria. This range of solutions were applied to 4 communal archaeological samples.

To test the efficiency and the feasibility of flotation in palaeoparasitology, 5 g of sediment sample was mixed with 20 ml of MilliQ-water and then centrifuged for 5 minutes at 400 G to form a sample pellet. Floating debris was then discarded along with the removal of the supernatant. 5.5 ml of of MilliQ-water was added to each soil sample before 1 ml aliquots were mixed with 5 ml of a flotation solution (the additional 500 μl was retained for microscopy). The 20 tests were then submitted to a second centrifuge spin. This run was for 10 minutes at 50 G and the centrifuge was set to decelerate rather than halt the run so the density gradient was not disturbed. Plastic pipettes were modified to increase the surface area of the tip and these were used to remove 1 ml from the surface layer of the gradients. When there was a visible distinction, the mid layer was also separated from the pellet. As a washing step, 10 ml of MilliQ-water was then added to each of the separated layers and then spun at 500 G for 10 minutes. The resulting supernatant was then removed. 10 μl of each layer was used for microscopy to establish parasite density.

Results

None of the floatation mediums utilised consistently separated parasites to the upper 1 ml of solution, nor were all eggs forced into the pellet. The egg counts for each layer and for the unprocessed soil can be seen in Table 1. There was 1 case however where complete eggs were found in separate layers from the incomplete and broken eggs. For samples 283, separated using NaCl, the top gradient only had broken *Trichuris* eggs in them, whilst the pellet and mid layers contained complete *Trichuris* shells, see Figure 1. Whether the eggs broke during microscopy or earlier is unclear. As this result

was not replicated in the other samples, it is difficult to assess the significance of this finding.

To check that parasite eggs had not been lost to osmotic stress, the data for each layer was normalised using the egg counts from the unprocessed samples. In 8 of these tests, the combination of the egg count from the pellet, mid layer and top layer for each flotation medium resulted in more eggs than in the unprocessed sample, see figures 2-5. This result may be indicative of an uneven distribution of eggs between sub-samples or variability in the researchers capacity to detect eggs. Regardless, the extremely low numbers of eggs found in the top 1 ml of the solution gradient indicates that flotation is not a suitable cleaning step for ancient parasites.

Discussion

This result directly contradicts the successful flotation of modern parasites. There are two possible explanations. The first is that flotation is a lossy process. Parasite eggs may be variable in their ability to float however, due to the high numbers of eggs worked with in a modern setting, a proportion will always float. This explanation comes into question with the findings of Sawitz (1942). This study quantified the percent of eggs at the surface layer, the upper and lower layers of the supernatant and in the sediment. They assessed specific gravity of nematode eggs when there was only 0-2 % remaining in the pellet and the rest in the surface layer. This indicates that these densities did not just float a portion of eggs. The most likely conclusion is therefore that taphonomic processes are at work. Helminth eggs can develop normally in hyposmotic or hyperosmotic solutions (Wharton 1980) therefore the lack of success in the flotation experiments could be due to taphonomic changes to the density of the eggs rather than due to osmotic stress destroying the eggs. Alternatively, the taphonomic changes may increase the permeability of the internal lipid layer, causing damage in flotation experiments. There has been no study on how nematode eggs change structurally or chemically over time, and it can be assumed that this is likely to be inconsistent between different environments. The idea that differential taphonomic processes may be at work is supported by the difference in success by other published authors. Reinhard et al. in 1986 and 1987 found that a range of flotation solutions (zinc chloride, zinc sulfate, formalin-ether, sucrose solution, and sodium chloride) were ineffective for separating ancient eggs from soil whilst Søe et al. (2015) used sugar/salt flotation to successful separate intact shells. If taphonomy is the key variable in the success of flotation, then this technique will remain useful for palaeoparasitologists but this pilot study suggests it should be used with caution.

Table 1: A table showing the number of total eggs identified through microscopy. Samples either represent one of six flotation solutions (none, saturated salt- NaCl, saturated sugar- Suc, saturated Magnesium Sulphate- MgSO, Glycerol- Gly and Percoll- Perc) and are either the top, middle or pellet layer. When the middle and pellet layers couldn't be distinguished it has been indicated. This was repeated four times (samples 198, 265, 283, 367).

	198	**265**	**283**	**367**
No float	8	16	10	8
Suc Pel	8		5	7
Suc mid	4		3	6
Suc pel/mid		4		
Suc Top	0	1	0	0
MgSo Pel	7	3	3	6
MgSo Mid	11	5	1	3
MgSo Top	0	2	1	1
NaCl Pel	6	3	6	8
NaCl Mid	1	1	2	3
NaCl top	0	0	2	0
Perc pel	17		3	1
Perc mid	11		3	0
Perc pel/mid		4		
Perc top	0	0	1	1
Gly Pel				5
Gly mid				4
Gly pel/mid	16	4	2	
Gly top	0	2	1	1

Figure 1: An image of the broken *Trichuris* egg in the top layer of NaCl flotation of sample 283.

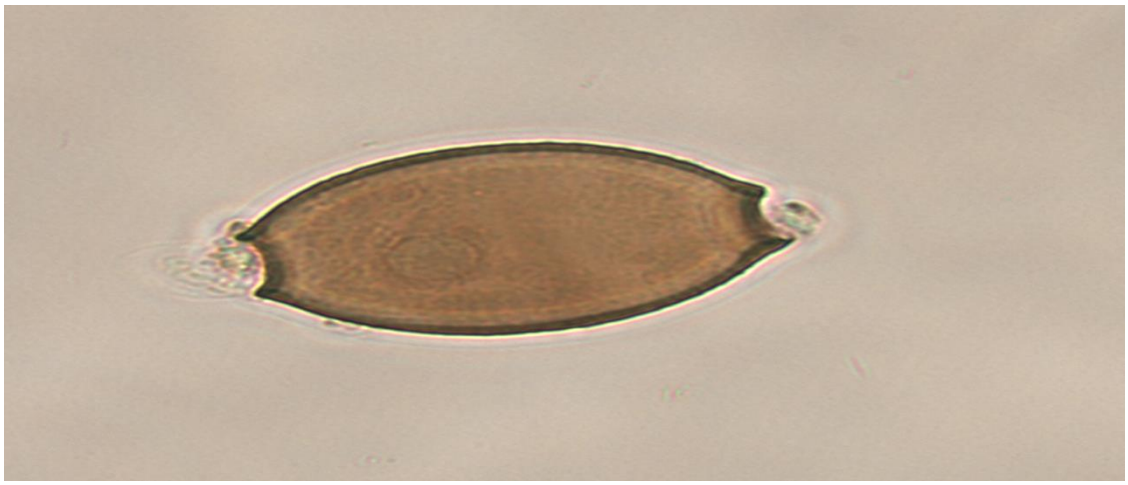


Figure 2-5: Four graphs, one for each soil sample, with the egg counts from each flotation solution normalised to the egg count from the unfiltered soil sample.



Extraction of single grave samples

Considering the success of the targeted approach in the published literature and during the shotgun versus targeted sequencing test, the original intention was to apply targeted PCR to samples from Chapter 3 (English graves). Samples that were identified as positive during microscopic analysis, both communal and single grave, were selected for extraction along with randomly selected negative samples (about 20% of negative samples per site). This was going to be used to compare the number of positive helminth identifications when using both microscopy and genetic approaches. One unexplored idea within palaeoparasitology is that one of these techniques may be better suited for helminth detection depending on substrate type or time period. 168 soil samples were therefore extracted following the protocol in Flammer (2018). See the table below. Of these, 26 were amplified using the Trichuris ITS1 primer used in this study and developed by Flammer (2018). As the gel runs did not look positive, the sequencing of these samples was not prioritised and is yet to be completed.

Sample code	Site Code	Microscopy results	Trichuris PCR?	Sample code	Site Code	Microscopy results	Trichuris PCR?
83512	YKHG	Positive		**8030 1/3**	DVCH	Positive	Y
83578	YKHG	Positive		**8030 2/3**	DVCH	Positive	Y
83582	YKHG	Positive	Y	**8064.1**	DVCH	Positive	Y
83678	YKHG	Positive		**8064.2**	DVCH	Positive	Y
83710	YKHG	Positive		**6768 1/2**	DVRS	Positive	Y
83914	YKHG	Positive		**6769**	DVRS	Positive	
84023	YKHG	Positive		**6769**	DVRS	Positive	
84032	YKHG	Positive		**6773**	DVRS	Positive	
84330	YKHG	Positive		**6790a**	DVRS	Positive	
84342	YKHG	Positive		**6790b**	DVRS	Positive	
84357	YKHG	Positive	Y	**6853**	DVRS	Positive	
83834	YKHG	Positive		**7111**	DVRS	Positive	
84422	YKHG	Positive	Y	**7116**	DVRS	Positive	
84428	YKHG	Positive		**7147**	DVRS	Positive	Y
84431	YKHG	Positive		**7148**	DVRS	Positive	
84536	YKHG	Positive		**5166**	IPS	Positive	
84557	YKHG	Positive		**5216**	IPS	Positive	
84560	YKHG	Positive		**5630**	IPS	Positive	

Sample code	Site Code	Microscopy results	Trichuris PCR?	Sample code	Site Code	Microscopy results	Trichuris PCR?
5829	IPS	Positive		**<376>**	CBPG	Positive	
20082	IPS	Positive		**3410**	CBPG	Positive	
20105	IPS	Positive		**3695**	CBPG	Positive	
20193	IPS	Positive		**3480**	CBPG	Positive	
20228	IPS	Positive		**3753**	CBPG	Positive	
20255	IPS	Positive		**3065**	CBPG	Positive	
20402	IPS	Positive		**3502**	CBPG	Positive	
20639	IPS	Positive		**3080**	CBPG	Positive	
20861	IPS	Positive		**3432**	CBPG	Positive	
20880	IPS	Positive		**3835**	CBPG	Positive	
20909	IPS	Positive		**3625**	CBPG	Positive	
251	ODDK	Positive	Y	**52**	WCH	Positive	
1164	ODDK	Positive	Y	**82**	WCH	Positive	
2172	ODDK	Positive	Y	**85**	WCH	Positive	
5422	ODDK	Positive	Y	**107**	WCH	Positive	
1896	ODDK	Positive	Y	**130**	WCH	Positive	
1910	ODDK	Positive	Y	**165**	WCH	Positive	
4177	ODDK	Positive	Y	**170**	WCH	Positive	
1702	ODDK	Positive	Y	**197**	WCH	Positive	Y
2358	ODDK	Positive	Y	**199**	WCH	Positive	
1036	ODDK	Positive	Y	**3021**	STJ	Positive	
3682	CBPG	Positive		**3027**	STJ	Positive	
3402	CBPG	Positive		**3045**	STJ	Positive	
3743	CBPG	Positive		**3095**	STJ	Positive	
3135	CBPG	Positive	Y	**2032**	STJ	Positive	
3658	CBPG	Positive		**2052**	STJ	Positive	
3931	CBPG	Positive		**2111**	STJ	Positive	
5079	CBPG	Positive		**2119**	STJ	Positive	
3674	CBPG	Positive		**2141**	STJ	Positive	
3490	CBPG	Positive		**4010**	STJ	Positive	
3529	CBPG	Positive		**4012**	STJ	Positive	
3448	CBPG	Positive		**3045**	PKS	Positive	
3644	CBPG	Positive		**5064**	PKS	Positive	
3390	CBPG	Positive		**3110**	PKS	Positive	
3978	CBPG	Positive		**5122**	PKS	Positive	Y
5167	CBPG	Positive		**1**	AMX	Positive	
3755	CBPG	Positive		**6**	AMX	Positive	Y
2524	CBPG	Positive		**7**	AMX	Positive	Y
2588	CBPG	Positive		**8**	AMX	Positive	Y
3611	CBPG	Positive		**21**	AMX	Positive	Y

Sample code	Site Code	Microscopy results	Trichuris PCR?	Sample code	Site Code	Microscopy results	Trichuris PCR?
3686	CBPG	Positive		**116**	SSSM	Positive	
3275	CBPG	Positive		**1170**	BUL	Positive	
521	LKHc	Positive		**2**	AMX	Negative controls	
529	LLS	Positive		**83806**	YKHG	Negative controls	
20677	IPS	Negative controls		**84255**	YKHG	Negative controls	
5338	IPS	Negative controls		**84419**	YKHG	Negative controls	
6772	DVRS	Negative controls		**84638**	YKHG	Negative controls	
6798	DVRS	Negative controls		**2579**	CBPG	Negative controls	
41	WCH	Negative controls		**3752**	CBPG	Negative controls	
100	WCH	Negative controls		**3698**	CBPG	Negative controls	
66	WCH	Negative controls		**3680**	CBPG	Negative controls	
119	WCH	Negative controls		**3571**	CBPG	Negative controls	
142	WCH	Negative controls		**3544**	CBPG	Negative controls	
227	WCH	Negative controls		**3364**	CBPG	Negative controls	
194	WCH	Negative controls		**3271**	CBPG	Negative controls	
166	WCH	Negative controls		**5237**	CBPG	Negative controls	
89	WCH	Negative controls		**3771**	CBPG	Negative controls	
62	WCH	Negative controls		**3394**	CBPG	Negative controls	
49	WCH	Negative controls		**468**	LKH	Negative controls	
123	WCH	Negative controls		**531**	LLS	Negative controls	
73	WCH	Negative controls		**8203**	DVCH	Negative controls	
3013	STJ	Negative controls		**4017**	PKS	Negative controls	
3029	STJ	Negative controls		**4089**	PKS	Negative controls	
3091	STJ	Negative controls		**5093**	PKS	Negative controls	
2019	STJ	Negative controls		**5107**	PKS	Negative controls	
4016	STJ	Negative controls		**8074**	DVCH	Negative controls	

Data analysis development

For the preliminary shotgunning pilot study, the BLAST assessment was originally run against a database consisting only of sequences downloaded from Wormbase (https://parasite.wormbase.org/index.html). Whilst positive matches were identified, it was quickly realised that this approach produced false positives. When the positive helminth reads were run through the online BLAST portal (https://blast.ncbi.nlm.nih.gov/Blast.cgi), there were stronger matches to other taxa. This approach to data analysis was therefore discarded.

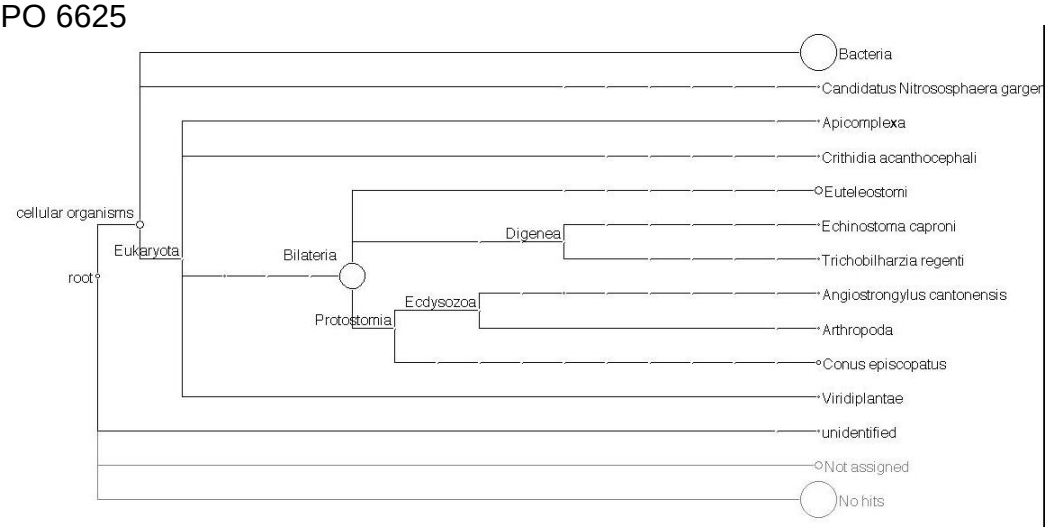
Fast X clipper was also originally used for data processing but the efficiency of this tool was bought into question, due to unbelievable hits, such as species only found in specific, Non-European locations (e.g. Tibetan antelope). Upon further research this step was completed using trimmomatic instead.

The data was also originally run through usearch cluter otus to both filter out chimeras and produce taxonomic units. After losing a high proportion of reads it was identified that this tool is inappropriate for shotgun data as the clustering protocol was designed for reads from a generic target amplification (such as 16S) so was far too stringent. Many metagenomic approaches use a clustering approach based on a predefined reference database, usually for microbial communities. Due to the incomplete nature of the helminth reference databases, the clustering step was omitted.

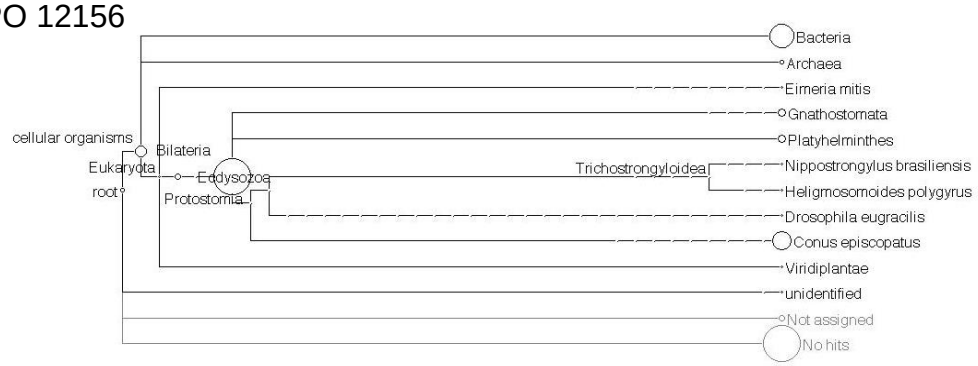
Finally, many of the BLAST hits, particularly of helminths, had long polyA tails at the ends of the reads. After clustering using usearch otus command, the poly A reads were removed manually in geneious. When this step was removed the number of reads that needed trimming was significantly higher and the manual approach was no longer appropriate. Instead the UrQT toolkit was identified and used instead.

5.10.4 BLAST matches, trees have been collapsed so that the helminths can be seen more clearly. Sample name is placed on each graph.

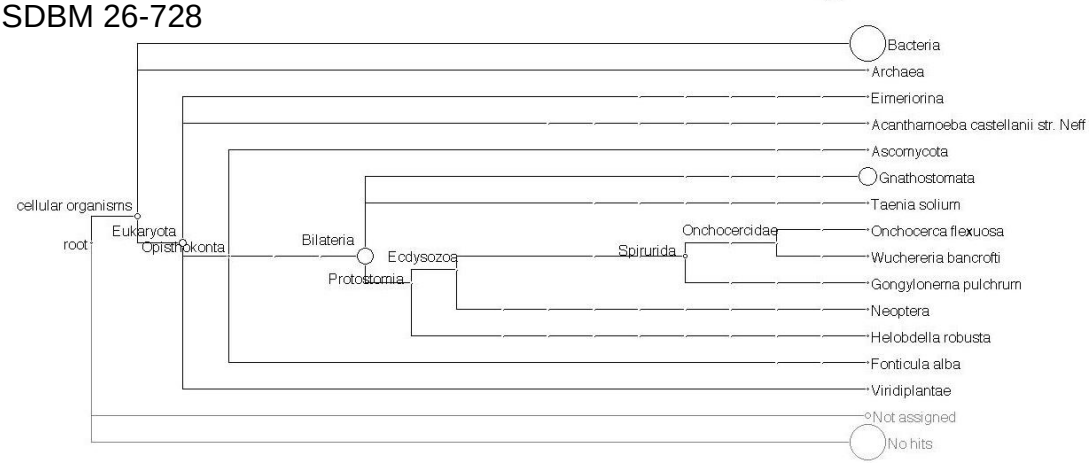
ZPO 6625



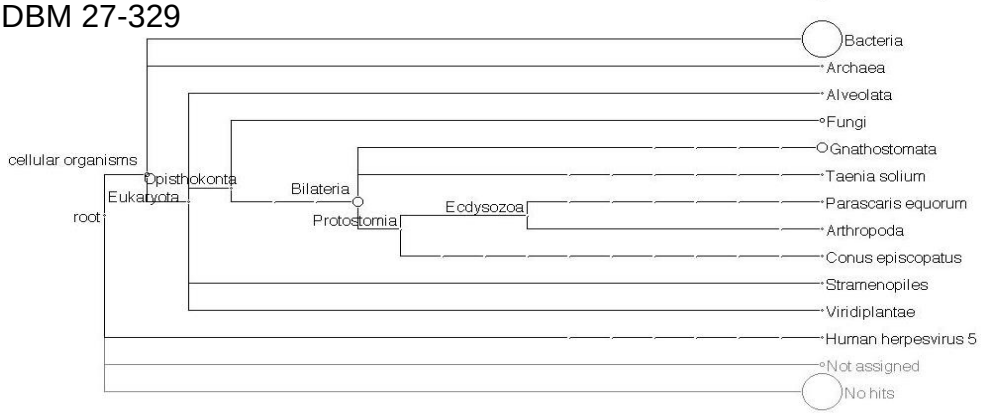
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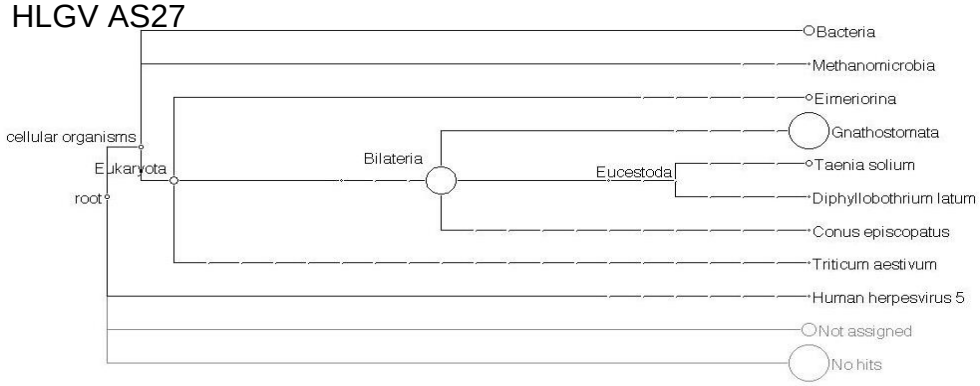
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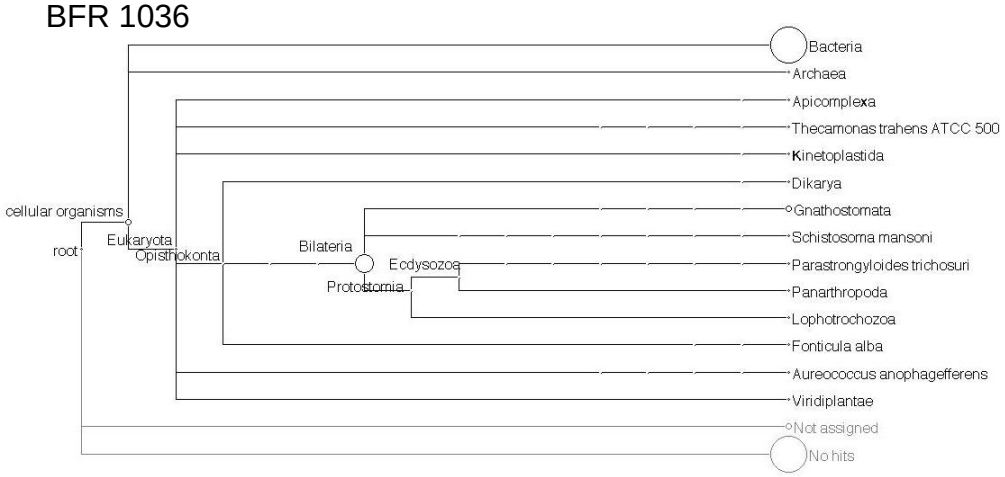
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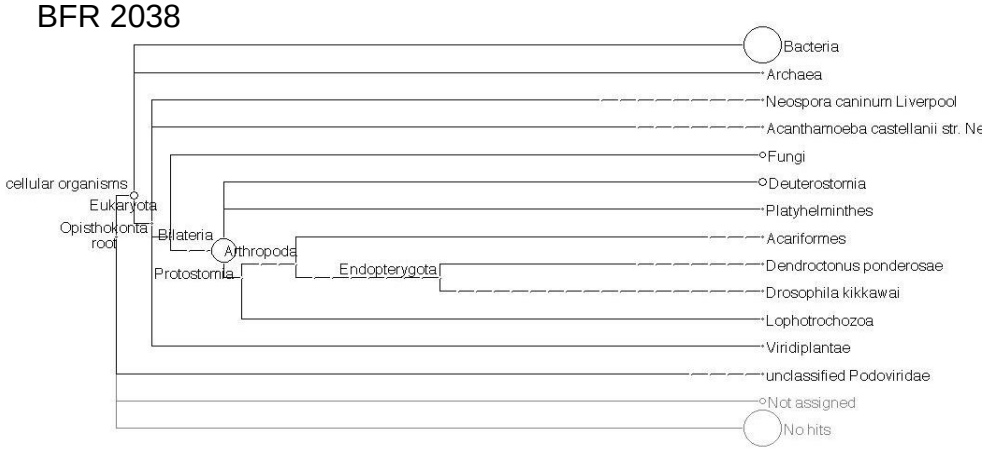
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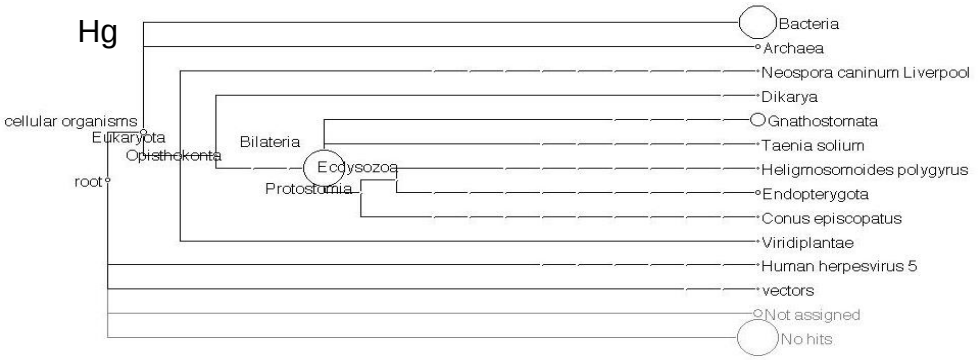
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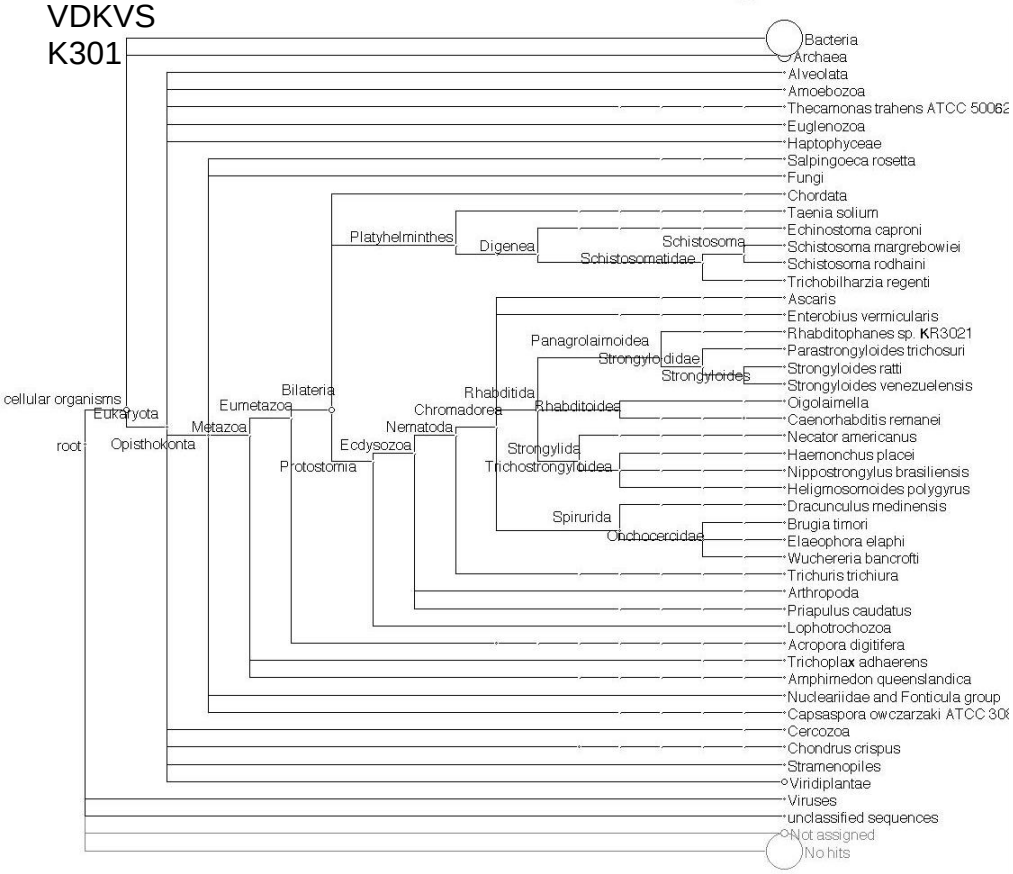
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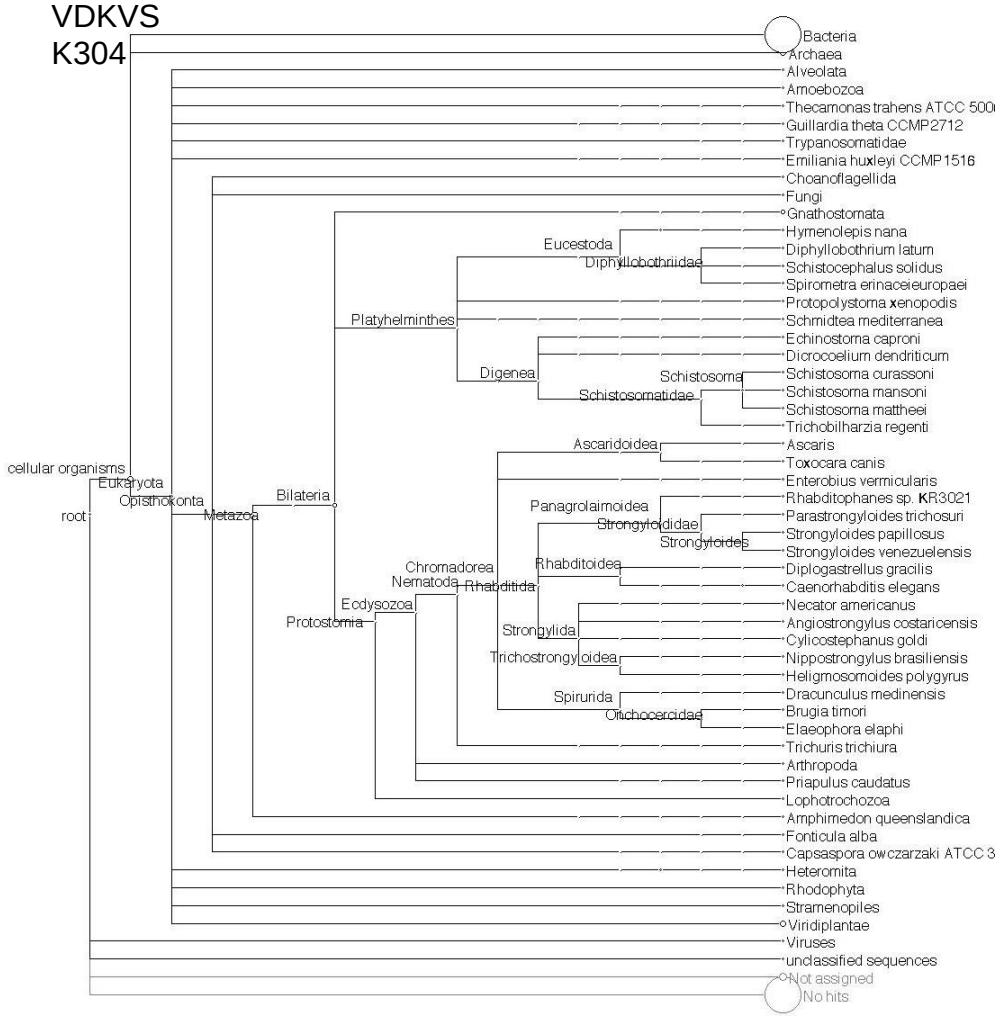
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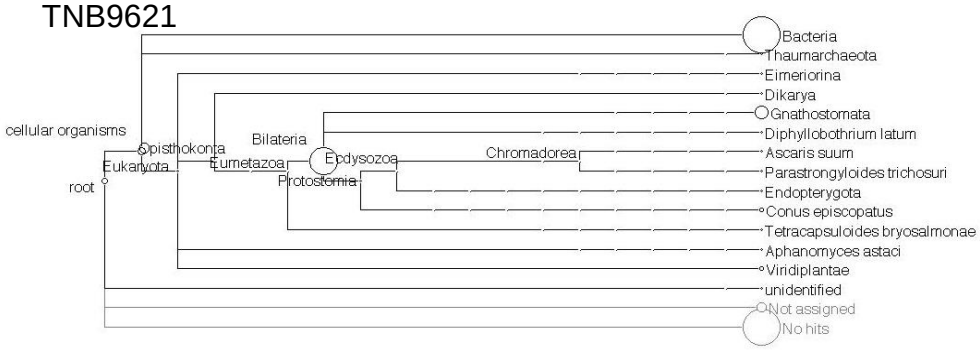
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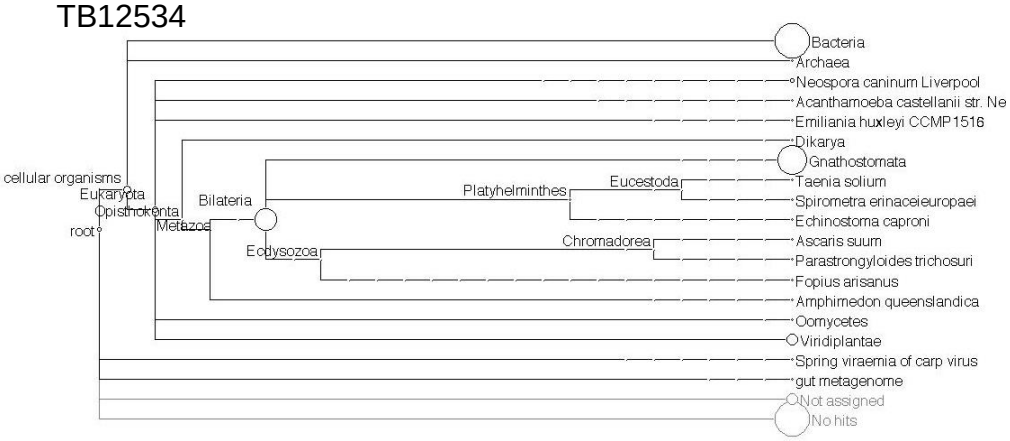
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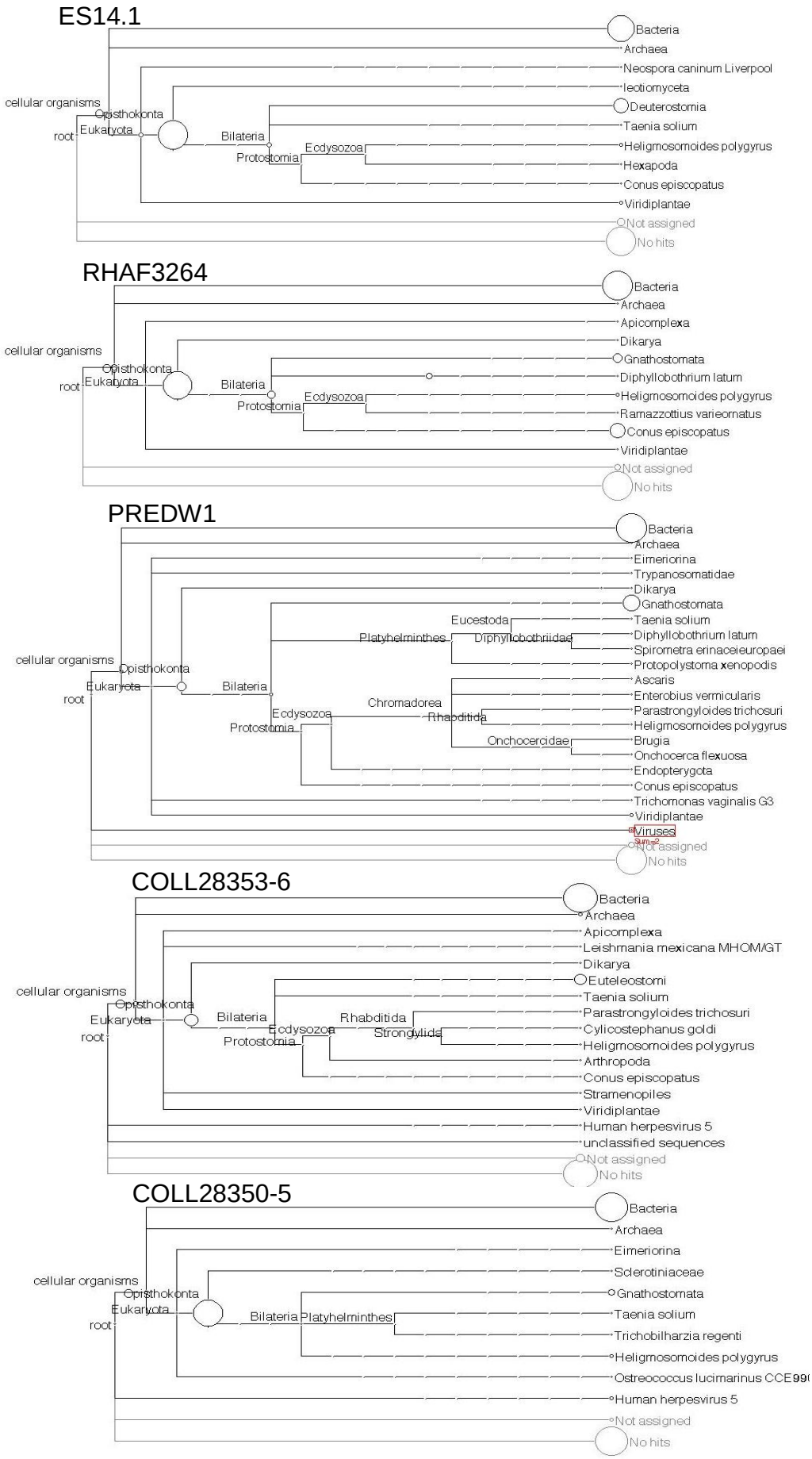


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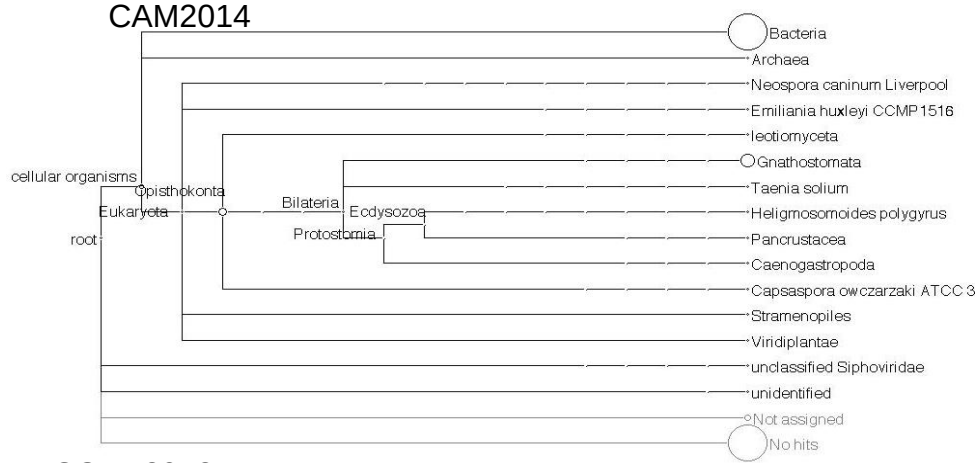


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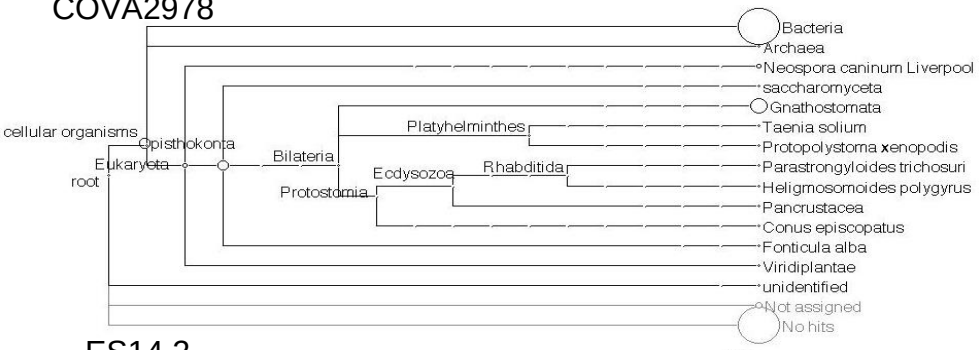



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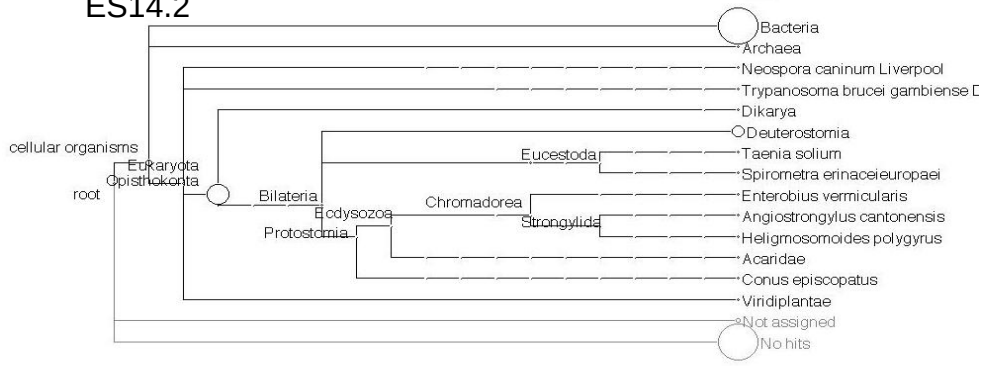
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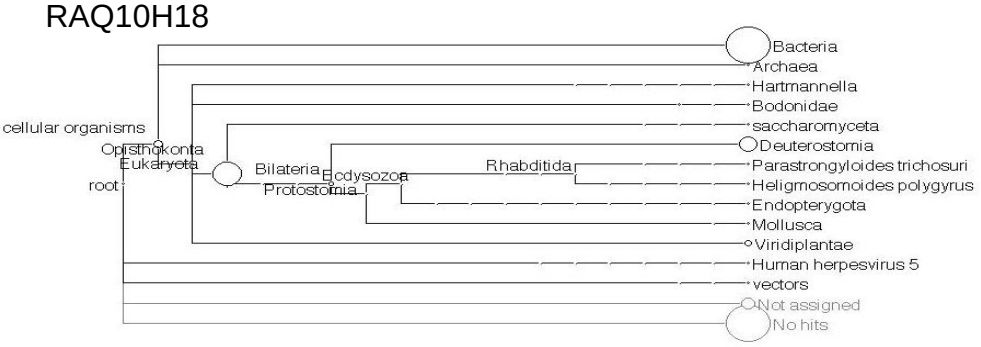
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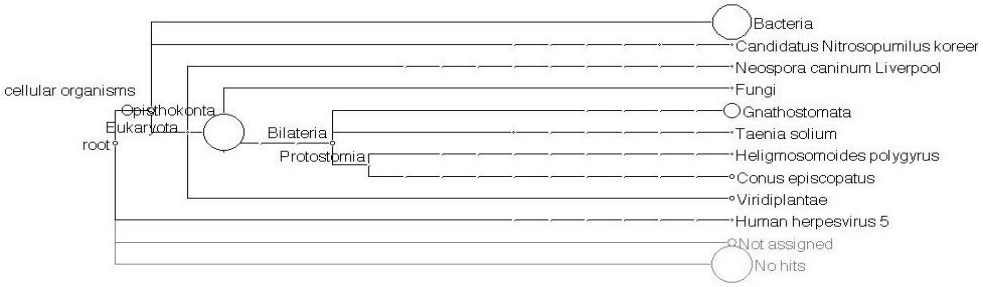
ES14.2



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TEM1



5.11 Permission from co-authors.
I hereby give permission to Hannah F Ryan to use our joint work as contribution towards her D.Phil. thesis to be submitted for examination at Oxford University.

I confirm that to the best of my knowledge, the author contribution statement below is accurate and Hannah F Ryan's contribution towards the work is greater or equal than that of any other co- author.

Chapter author contribution statement: H.R., P.F., J.H., A.S. and G.L. designed the research; P.F., G.L., A.P., and H.R. collected samples; P.F., A.P. and H.R. performed microscopic analysis; P.F. provided samples and carried out the genetic extractions for the samples used for preliminary Shotgun test and primer test runs, H.R developed and carried out the genetic extraction tests of the target vs shotgunning samples with input from P.F; H.R. ran targeted PCR and gel electrophoresis with input from P.F.; H.R. carried out the HiSeq library build; P.F. carried out the MiSEq library build; H.R. designed the variable *Ascaris* Primers with input from P.F., G.L., A.S.; P.F. designed all other unpublished primers with input from A.S.; L.F. and S.P. developed and carried out demultiplexing pipelines, H.R. carried out other data cleaning steps, H.R. carried out shotgun analysis and BWA alignments; A.S. and G.L. secured funding for the project; H.R. wrote the initial draft of the manuscript with input from P.F., A.S. and G.L.

Date: 17/4/2019
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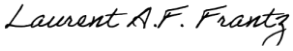
Date: 26/4/2019
Name: Adrian Smith



Date:25/4/2019
Name: Patrik Flammer



Date: 15/4/2019
Name: Laurent Frantz



Date:17/4/2019
Name: Stephen Preston



Date:15/4/2019
Name:James Haile



Date: 23/4/2019
Name: Angela Perri



Chapter Six: Discussion and Conclusion

6.1 Review of the goals of this thesis

This thesis set out to establish whether palaeoparasitology had the potential to move beyond species identification, to quantifying the presence of helminths in archaeological contexts and reconstruct their population dynamics using ancient DNA. It was apparent from the literature, that without these two approaches in palaeoparasitology, discussion of the health impact of helminths would remain limited. The lack of dialogue in this area was inconsistent with the pathological significance of helminths in modern populations. The ultimate goal for studying pathogenic organisms in the past is to make quantified statements of their impact on health in different environments and cultures, so a better understanding of the resilience and weaknesses of these pathogens can be developed.

For microscopy, the focus was on large scale analysis of individual burials to assess whether they could be used as a proxy for establishing prevalence rates of helminth infection in past societies. The majority of published palaeoparasitological studies have been on communal contexts where there is no clear relationship between the eggs present and the human hosts (Jones, 1985), and there are differences in the deposition and retention of helminths eggs depending on the context (De Rouffignac, 1987; Fisher et al., 2007). Of the published studies that have utilised single burial samples, the majority were small in scale and the largest study identified in the literature review was on 75 samples from one site (Seo et al., 2010). This thesis aimed to exceed those that had previously been published in terms of scale on both temporally and spatially distinct populations. This provided robust data to assess the biological and environmental factors that could be responsible for risk of infection in past societies.

For genetics, two areas were identified for improvement, which would build upon the steps described by Flammer et al. (2018) and Søe et al. (2018). The first was to establish the feasibility of utilising museum samples for the production of a ‘near recent’ database of the most commonly researched helminth genomes in areas that are no longer endemic. With a more complete reference database for helminths, the capacity to infer the continuity and spread of these pathogenic organisms between populations would be more robust. The second goal was to develop and compare different genetic approaches to palaeoparasitology in sedimentary samples, to establish which approach has the greatest capacity to explore questions of phylogeography and population dynamics in these taxa. Whilst this area is starting to be explored using *T. trichiura*

(Flammer et al., 2018), this approach should be applied to other species of helminth as they would provide a complimentary dataset.

All of the methodological approaches that we identified as lacking from palaeoparasitology have been explored and their strengths and weaknesses identified. In this chapter we explore what these methods can tell us about the impact of helminths on health in past populations. The future direction of palaeoparasitology is to build on the developments made here so that the health impacts of helminths in historical populations can be routinely researched and discussed.

6.2 Key findings of wide scale microscopic analysis of single graves

6.2.1 Reliability of single graves for reconstructing prevalence rate

Archaeological sciences are plagued by issues of preservation and contamination and this holds true for palaeoparasitology. One of the concerns when beginning this study was that the quantification of helminths from single burials would be too low to detect any significant patterns in the dataset due to the low density of eggs recovered from these contexts. For example, in Seo et al. (2010) only 7 out of 75 graves had evidence of helminths, and all of the positive burials were unusually well preserved to the extent that clothes, hair and brain tissue still remained. Alternatively, as helminths can be found in environmental samples, there was the risk that helminths would be over represented in skeletal remains due to external contamination. It is also of note that in our study, the samples were from Europe which excludes the danger of modern contamination. For research in endemic areas, this issue would need to be factored into the research design.

The recovery of *Trichuris trichiura, Taenia* spp. and *Diphyllobothrium latum* were all low which made it difficult to identify patterns in the prevalence rates of these species between or within sites however, *Ascaris* spp. were recovered at a much higher rate. As the average *Ascaris* spp. prevalence rates found within the archaeological sites studied in both Chapter 2 and Chapter 3 closely overlapped with those found in modern epidemiological studies, this suggests that there should be a degree of confidence in the reliability of quantifying helminths using single burials. In addition, the archaeological sites studied in Chapter 2 (which was the largest of the two microscopic studies in this thesis) uncovered that children had a higher prevalence of helminth infection than adults, as is found consistently in modern epidemiological studies (Bethony et al., 2006). The ability to detect a well-established risk factor in archaeological samples suggests that contamination and degradation of helminths over time is not enough to mask patterns of infection.

One anomaly identified in this thesis, which requires further research, is the discrepancy between the prevalence of *T. trichiura* in single graves versus communal contexts. As discussed in Chapter 1, in published studies looking at communal contexts *T. trichiura* were found in higher concentrations than *Ascaris* spp.. This trend was strong enough for some authors to suggest that a high number of *Ascaris* spp. eggs indicated non-human waste. Of the studies where the types of helminth recovered are known and not summarised by Kenward (2009), 21/23 had *T. trichiura* present whilst only 16/23 had *Ascaris* spp. present (see Section 1.12.1). Meanwhile, in both Chapters 2 and 3 *Ascaris* spp. was found at a much higher rate than *T. trichiura.* Whilst *T. trichiura* were present for all sites in Chapter 2 (all Medieval), in Chapter 3 they were absent from 14/19 skeletal groups and there was no evidence of this taxa in the Prehistoric and Roman groups.

Ascaris spp. release around 200,000 eggs a day whereas *T. trichiura* release between 3,000-20,000 eggs and therefore you would expect a higher proportion of *Ascaris* spp*.* eggs deposited in the archaeological record. In addition, the lack of *T. trichiura* in the oldest single grave contexts in this thesis suggests that these eggs are less resistant to diagenesis. Finally, *Ascaris* spp. infections are more common in modern population than *T. trichiura* (Pullan et al. 2014) so one may also expect a higher proportion of *Ascaris* spp. in the archaeological record if this pattern has been consistent over time. By combining these points one would expect that *Ascaris* spp. should be more commonly recovered from all archaeological sites however, this is not the case for communal contexts. A study on the coinfection of these two parasites in both communal samples and single graves for different time periods and climates may address the role of differential degradation in this discrepancy.

In Chapter 2 all cases of coinfection occur in the Medieval period due to the limits of the study, and the three cases of coinfection of *Ascaris* spp. and *T. trichiura* in Chapter 3 also date to the Medieval period. An assessment over time is therefore not feasible with the single graves data in this thesis. Meanwhile, *T. trichiura* and *Ascaris* spp. have been found to coinfect in communal deposits in all time periods. In Chapter 1 there were only two cases where *Ascaris* spp. eggs were found but *T. trichiura* was absent and again, these both date to the Medieval period. The absence of *Ascaris* spp. on the other hand occurred in sites dating from the Prehistoric through to the Medieval period (there were no records from the Industrial period). It is difficult to draw any conclusions about whether time effects the propensity for these helminths to coinfect with the data as it stands, however an increase in the number of single graves analysed and a deeper dive into the literature would resolve this question.

Whilst the number of helminths in single graves can only ever be used as a proxy for the true prevalence rates, this thesis has established that wide scale single burials analysis is a fruitful area for producing prevalence data that can be compared and contrasted between sites. This is because single graves are a more consistent context (in terms of the deposition and retention of helminth eggs) in comparison to environmental samples or latrines. This thesis included the analysis of 871 single burial samples, from 26 archaeological sites spanning from the Neolithic through to the Industrial period, and from three different countries. This is more than 10 times larger than the previous biggest published study (75 graves in Seo et al. (2010)) and represents a sharp contrast from the studies of 25 individuals or fewer (Anastasiou et al., 2018, n=25; Carrott et al., 1996, n=4; Dittmar and Teegen, 2003, n=1; Jaeger et al., 2013, n=10; Jones, 1986, n=1; Le Bailly et al., 2014, n=3; Mitchell et al., 2013, n=1). It is also the first study to focus on comparing the palaeoparasitological analysis of single burials from different archaeological sites. Not only does this result in robust prevalence rates from multiple different historical populations, it means that we have the capacity to compare these sites and time periods to investigate differences in the cultures and environment that would impact the helminth population.

6.2.2 Individual Risk Factors

The advantage of being able to quantify the prevalence of helminths is that we can begin to explore risk factors systematically. The most obvious to explore when working with skeletal remains are age and sex. In modern epidemiological studies children are at a greater risk of helminths (in terms of prevalence, intensity of infection and associated pathology, Bopda et al., 2016; Bundy, 1995) whilst sex only becomes a risk if there is a division of behaviour that increases exposure in one group (Brooker et al., 2004; Gildner et al., 2016; Trang et al., 2007). When the data was combined for sites in Chapter 2, and the sites in Chapter 3, sex was not identified as a risk factor however, there was variation identified within sites (Chapter 2) and over time (Chapter 3). At the site level, the biggest differences in prevalence rates in association with sex were identified for *T. trichiura, Taenia* spp. and *D. latum,* the latter two of which may indicate a difference in diet between the sexes. This thesis therefore highlights another tool for tracing sex-based behavioural differences in the archaeological record, something that is notoriously difficult to do.

Meanwhile when the data was combined, children and young teenagers were identified to be at greater risk of helminth infection in Chapter 2. This group are believed to be at a higher risk either due to differences in behaviour or due to an under-developed

immune response (Martins-Melo et al., 2017; Truscott et al., 2014). The consistency of the higher risk of helminth infection in children over time may put more weight behind a biological explanation rather than a behavioural one, but this point is purely speculative. The same pattern of increased risk in young individuals was not detected in Chapter 3. It is of note that total number of samples studied (Chapter 2= 589, Chapter 3= 463) and the number of skeletal individuals identified as under 18 (Chapter 2=122, Chapter 3=71) was larger for Chapter 2 than Chapter 3. The data was also subdivided into a greater number of age groups in Chapter 3 (seven, rather than four) which may also have impacted the ability to trace risk in association with age.

6.2.3 Environmental Risk Factors and change over time

The ability to assess prevalence between time periods is important for comparing and contrasting behavioural and environmental differences between the periods which could impact helminth prevalence rates. When assessing the published literature of helminths over time in the UK, the most reliable inference that could be made was that helminths were found consistently in communal samples regardless of time period (Chapter 1). The results of Chapter 3 supported this finding however, it also highlighted that there were fluctuations in the prevalence of helminths over time that has previously gone undetected. WASH (Water and Sanitation for health) practices are often cited as a strong risk factor for helminth infection (Campbell et al., 2018) and the "sanitary reform" of the mid 19th century is a clear behavioural and environmental shift that would lead to the decline in soil transmitted helminths (STH). The dip in prevalence rates of *Ascaris* spp. during the Industrial period, identified in Chapter 3, has provided evidence to support this theory, and also highlights how quickly those cultural changes impacted the helminth population.

There are however, a variety of behaviours and infrastructural changes that fall under the umbrella term of WASH. Gass et al. (2014) found that by separating some of these factors out and assessing their significance using recursive partitioning approaches, that some behaviours and structures were more significant to determine risk of infection than others. In their study this included the factors: whether there was a latrine at home or at school, knowledge on hand washing and water supply treatment, latrine conditions, access to hand washing and drinking water at school, and soil eating behaviour.

The variability of the impact of different hygiene practices can explain why the Roman period, which is perceived as sanitary because of the public washing facilities, sewer systems and piped drinking water, was still found to be endemic for helminths.

This held true for archaeological sites across the Roman empire, possibly due to mitigating behaviours such as unclean communal facilities and the use of human faeces as fertiliser (Mitchell, 2016). This study was supported by the results of Chapter 3 where a high prevalence rate of helminths was detected in the Roman period. Meanwhile a surprisingly low prevalence rate was detected in the Anglo-Saxon period, which is not renowned as a period of good sanitation (Bonser, 1944), suggesting that other factors were influencing the prevalence of helminths at this time.

The Anglo-Saxon period has limited archaeological evidence in comparison to the Roman and Later Medieval periods, so it is at the centre of a number of archaeological debates around migration (Hills, 1990) and settlements patterns (Hamerow, 1991). It is increasingly becoming apparent however, that the period is characterised by a drop in population size, non-nucleated settlement patterns, regularly shifting farmsteads and temporary structures, whereas the Roman and Later Medieval periods had large permanent populations centred around key urban centres (Astill, 2011; Hamerow, 1991, 2011, 2012; Wright, 2015). With temporary and/or dispersed populations you might expect a reduction in waste build-up, and therefore a reduction to the rate that the population would come into contact with it. Both of these factors are key for the spread of soil-transmitted helminths.

Notwithstanding the fact that there will be clear differences in the size and density of settlements in any time period, this broad change in the population during the Anglo-Saxon period may have had a material impact on the population of urban centres that did exist (including London, Bath, Cirencester and Lincoln; Lacey & Danziger (1999)). This suggests that rather than hygiene practices, permanence and density of populations currently appears to be the most significant indicator of helminth infection load in historical Britain, that is, until centralised powers had the capacity, knowledge and drive to build sanitary infrastructure that was efficient enough to deal with the waste build-up of permanent urban areas.

6.2.4 Inferences for modern epidemiology

An ability to compare between time periods means that we can also compare the significance of helminths in historical and modern populations. This was explored in Chapters 2 where samples from the Medieval Period were utilised to produce a baseline level of infection prior to the implementation of modern WASH practices and the development of effective chemotherapeutic agents. Whilst the average *Ascaris* spp. prevalence rate from Medieval Europe was significantly different from the average prevalence rate of endemic regions after the WHA 54.19 resolution (Pullan et al., 2014),

there were some modern endemic regions, even after the WHA54.19 resolution, that had a prevalence rate that was similar to those recovered from archaeological sites. This suggests that there is a degree of regional variation in the implementation of modern interventions for the treatment of helminths. The medieval prevalence baseline developed in Chapter 2 could therefore be useful for researchers aiming to identify whether the interventions being used in different regions are effective, especially if there has been no previous research on the prevalence rates for that region.

One of the reasons to study pathology and infective agents in the past is that these historical populations provide case examples of how changes in culture and environments, impact of the prevalence of helminth. This can be used to infer information that can be used for health practices and policies today. For example, in Chapter 3 the lack of impact that hygiene practices had on the prevalence of helminths until the Industrial period, suggests that only a wide-scale approach to sanitation has any significant impact on the helminth population. This result also indicates that we do not need the full suite of changes, including chemotherapy, undertaken in modern eradication examples such as Japan and the Republic of Korea (Hong et al., 2006; Kobayashi et al., 2006) for there to be a decline in the prevalence of helminth. With more targeted research on sites with clear archaeological or historical evidence of sanitation, the effect of the different individual practices may begin to be dissected. This information could be utilised by modern policy makers when deciding which strategies are the most suitable and sustainable for mitigating the morbidity effects of helminth infections in modern populations.

6.3 Limitations of the approach in this thesis

The insights from this thesis would not have been possible with the analysis of latrines, or if analysis was restricted to burials within an individual site. This highlights the power of a systematic wide scale approach to single burials in palaeoparasitology. There is however, still room for improvement. Even with a large sample set, as soon as the data was divided to represent different demographics, time periods and/or geographic areas, the numbers began to diminish and the power to infer differences which were statistically significant began to drop.

This was particularly problematic for the cestodes (*Taenia* spp. and *D. latum*), because the recovery of these eggs was much lower than the STH. This was not necessarily a surprising find as these infections are less common in the modern population and less commonly recovered in archaeological sites in comparison to the STH. To ensure that the statements made were statistically supported, the focus of this

thesis was to evaluate broader trends. There are other ways in which health and helminths could be explored, as discussed below, however this would require increased sample sizes or targeted selection of archaeological sites that would be best suited for resolving different research questions.

6.3.1 Limits to the exploration of morbidity

One alternative way to explore prevalence data is to assess whether there is correlation in the recovery of helminth eggs with skeletal indicators of generalised stress (often associated with poor nutrition and health) including cribra orbitalia and enamel hypoplasia. Today helminth infections often result in mild or no symptoms, but they can contribute to malnutrition, diarrhoea and abdominal pain, general malaise and weakness, and impaired growth and physical development. A range of factors can result in skeletal indicators of stress, so a strong correlation of helminth infection with these skeletal abnormalities could suggest that helminth infections were a significant contributor to overall poor health within historical populations. Whilst this measure is not perfect, it would be interesting to investigate.

This analysis would have to be restricted to juveniles as the skeletal pathologies are either limited to, or more likely to, develop during childhood (Stuart-Macadam, 1985). In adults the link between these two indicators of health would be uncertain and therefore should be excluded for a conservative study. Unfortunately juveniles are regularly under-represented in archaeological sites (Kamp, 2001) which will limit the sample size. Samples analysed would also need to come with complete osteological analysis including pathological data. This is an expensive and time-consuming step which is not always carried out, particularly in cases of rescue archaeology. Finally, a lack of ova is not necessarily indicative of no infection during life, therefore a conservative approach would be to restrict the analysis to those which were positive for helminths by microscopy. Using these criteria for exclusion, of the 463 burials studied from the UK only 13 remained that could be utilised for this type of analysis. This highlights the value of both a larger dataset and one that comes with complete osteological and archaeological information. Many of the sites used in this study had either not been fully excavated or fully analysed which limited the capacity to undertake this type of analysis.

6.3.2 Limits to the exploration of environmental risks

Not only does the lack of contextual information restrict what assessments can be made of helminths in relation to the skeletal evidence, but, also how they are affected by the external environment and culture. Our study of the UK in Chapter 3 used broad time periods for analysis. For finer scaled inference of changes over time, the sites and

possibly even the individual skeletons would need to be dated by absolute means (for examples radiocarbon, luminescence, grave stones). As seen in Chapter 2, this is not impossible as we assessed change over time within two sites, Ellwangen and Ipswich, but these represent very good case examples rather than the standard (2 out of 22 sites studied in this thesis).

In Chapter 2 we explored helminth prevalence in relation to estimated population size and in Chapter 3 we discussed how overall trends in population structure over time was associated to the risk of helminth infection. What has not been fully explored in this thesis is the significance of rural or urban settlement type as a risk factor. This is a key topic in the literature of pathology in the archaeological record as urbanisation lead to a rise in a wide range of health problems (e.g. Gowland et al., 2018). To explore this as a risk factor the burials analysed would need to have clear connections to a settlement type, such as being buried within or adjacent to a domestic site. Whilst some of the sites studied for this thesis were clearly associated with urban centres, for others there was very little contextual information to work with (as described in the Supplementary to Chapter 3 (3.8.1)). This is a clear area for future research, but to address the significance of urban versus rural settlement types as a risk factor, the sampling strategy would need to be targeted to ensure that there was equal representation of these two settlement types.

6.4 Next steps to develop microscopy approaches

6.4.1 Data sharing to strengthen palaeoparasitology

Like with many other disciplines: the greater the amount of data, the greater the number of robust conclusions that can be drawn. Wide scale analysis, such as the studies undertaken for this thesis take a long time to develop, and a much larger number of sites and samples would be required for research into other risk factors not considered in this thesis, as outlined above. The most efficient way to produce a database of that size would be to collate different studies however, as was found in the overview of published work in the UK in Chapter 1, non-standardised reporting made it difficult for them to be combined in a meaningful manner.

Online databases where researchers upload their raw data is a tool that is already being utilised by different disciplines. For example, in the field of genetics there are two online databases (NCBI-GenBank and EMBL-EBI) where researchers upload the sequences of genomes they have published. The deposition of genetic data is often a requirement of publication which means that a vast quantity of information is freely available. These resources are vital for the comparison of sequences that have been

produced to those that are spatially or temporally distinct, or which come from other closely related organisms. There is no reason why the same principle could not be applied to palaeoparasitology.

Whilst there is variation in the data that can be retrieved from different sites, there should be some details recorded as standard so comparisons can be made readily. Some of the details that should be included would be starting soil weight, processing methodology, helminth species detected with accompanying images and number of ova detected. It would be useful to include if there was low confidence in the identification of a particular ova but this would be difficult to standardise. The skeletal information to include would be: sex (F, F?, ?, M?, M), age and pathology including the methods used for their determination, and indicators of status such as burial goods or location within the cemetery. Finally, contextual information would include: date, geographic region, urban or rural population and type of burial ground (such as monastic, poor house, hospital, ceremonial). A reference to both the palaeoparasitological paper and to site reports, available in a public database, would need to be included so other information can be easily recovered when necessary. The list of traits suggested here represent a starting point, so a database such as this would need to have the capacity to expand as the field develops.

6.4.2 Addition of the Enteric Protozoa

The utility of more data is not just restricted to the sample size or the number of sites studied, but also the scope of the research. This thesis was focused on helminths but enteric protozoa are an additional group of parasitic organisms that can be detected in archaeological sediments. These parasites are also a significant health issue in modern populations, particularly for those who are immunocompromised, and they are also spread by poor sanitation (Fletcher et al., 2012). They would therefore provide complimentary evidence to the study of helminths for interpretation of the health and hygiene of past populations. Enteric protozoa that have been detected in archaeological sites include the taxa: *Cryptosporidium* sp., *Giardia* sp., *Entamoeba histolytica*, and *Cyclospora* sp. This group of pathogenic organisms are underrepresented in the archaeological record in comparison to helminths because their cysts and oocysts are fragile microstructures which are less resistant to taphonomic processes so they are only rarely detected by microscopy (Frías et al., 2013). Instead, they are increasingly being traced utilising immunofluorescence (IFA) assays or enzyme-linked immunosorbent assay (ELISA) tests.

These techniques have been used successfully to recover signals of protozoans from coprolites (Morrow and Reinhard, 2016; Ortega and Bonavia, 2003), latrines (Le Bailly et al., 2008) and, individual burial samples (Le Bailly et al., 2006). It is of note that in some studies, formalin was added to samples to reduce the risk of bacteria and fungi growing which would compromise the validity of ELISA tests (Le Bailly et al., 2008). As discussed in Chapter 4, the addition of formalin would be problematic in cases where researchers also wanted to carry out ancient DNA analysis on the same samples. In our proposed database, any results from IFA, ELISA and/or genetic analysis for species identification would be useful additions to the microscopic data. With further research information beyond species identification garnered from biomolecular approaches, such as the spread of these pathogens, have the potential to also be included in an online database.

6.5 Key findings from the genetic approaches

6.5.1 The suitability of Fluid Collections for reference genomes

In Chapter 4, genetic approaches were used to confirm the genus of a helminth museum specimen that was part of the Oxford University Natural History's fluid collection. This chapter has shown that biomolecular approaches can be utilised on samples such of these, despite the problems associated with formalin fixation. One of the samples was confidently identified as *Macracanthorhynchus* using genetic data which suggests that the method used in this thesis can be applied to museum samples that are damaged for taxonomic identification.

The ultimate goal of this research however, was to identify whether museum fluid collections were an appropriate resource set to use to build a 'near recent' reference database of helminths from regions that are no longer endemic. One of the seven samples contained endogenous DNA and of the reads produced, there was partial coverage of the published nuclear ribosomal genes and no alignment to the complete mitochondrial genome. With the data as it stands, it appears that the utility of museum worms for a reference database would be prohibitively expensive and highly destructive for the amount of data that would be retrieved. To use fluid collections for a reference database, samples would need to be deep sequenced and for every data point of interest (for each species, time period and geographic region), multiple museum samples would be damaged to get a positive result.

The likelihood that a curator would be willing to agree to this process is low but, this chance could be improved in cases where the collection had a good record of the preservation history. For example, the Smithsonian fluid collections house multiple worm

specimens and their database notes that the museum initially used a preservative that "… was a solution of 70% ethanol, 3% formalin, and 2% glycerine" (Smithsonian National Museum of Natural History, 2019). On further investigation there may be notes not currently publicly available that describe the fixation processes as well. On the other hand, there may be a greater proportion of endogenous DNA in museum samples than the results currently suggest. As the genetic reference sequences for helminths are poor, some of the endogenous reads could go unidentified if the reads come from a locus not covered in the reference database. Considering the potential utility of fluid museum collections for the study of helminths, museum collections should be revisited in future studies when a collection with good storage records is identified and the number of genetic references for helminths increases.

6.5.2 The strength of different sequencing approaches on sedimentary samples

In Chapter 5 there were two key results established for others to build upon. The first is that shotgunning can be carried out successfully to identify helminths using small soil starting weights without utilising floatation on both communal and single burial samples. The power of shotgunning is that it is a non-discriminatory approach and therefore there is the capacity to identify species that would not be expected. For example, *Schistosoma mansoni* was identified in a communal latrine in Medieval Denmark despite the fact that it is naturally located in South America and the Caribbean, Africa, and the Middle East.

The identification of *S. mansoni* could represent a false positive match either because it is the most closely related species represented in the reference database or, a match stemming from incomplete and contaminated references. On the other hand, it could represent a true hit and provide insight into an unusual case example as *S. mansoni* which are very rarely recovered from archaeological sediments (Bouchet et al., 2002). *S. mansoni* require *Biomphalaria* snails to proliferate and while these are predominantly isolated to tropical and sub-tropical regions, a small number of *Biomphalaria tenagophila* have been found in Romania (Majoros et al., 2008). This suggests that *S. mansoni* could have the capacity to proliferate in Europe. Alternatively, the presence of *S. mansoni* could be indicative of trade networks between Medieval Denmark and regions where this species are still endemic. The low number of hits for *S. mansoni* however, meant that this was not further investigated for this thesis. This case example does however, suggest that interesting results could be uncovered if the approach developed in this thesis is applied more widely in palaeoparasitology.

The successful results from the shotgunning approach indicates that it is an appropriate tool for identifying unexpected species of interest but this would have to be followed by further investigation to confirm whether the hits were robust. A high frequency of C to T substitutions at the 5' end and G to A substitutions at the 3' end of sequences are typical nucleotide misincorporation patterns in aDNA, and this can be exploited to identify which sequence are modern contaminant. This is a conservative approach as not all aDNA molecules carry post mortem damage, but, the alternative would be to use tools that establish authentication by the ratio of derived and ancestral alleles using known variation across species and/or populations (Orlando et al., 2015). The latter approach would require significantly higher numbers of reference helminth genomes to be published to be feasible.

Chapter 5 also identified that the shotgunning approach did not always identify *Ascaris* spp. in samples that were positive for this species when utilising a targeted PCR approach. This suggests that in the current format, species identified by shotgunning represent a sub-sample of the complete helminth picture and therefore this approach is not the most appropriate tool for researchers who are investigating a single species. At very high sequencing depth shotgunning may have a detection rate equivalent to targeted PCR, however this would be prohibitively expensive for any large-scaled analysis. It is not necessarily a surprising find that a targeted approach is more sensitive than shotgunning on a mixed context, but, it has the clear drawback that it is a selective process. Both approaches have their merits, and this thesis has established that the selection of the most appropriate tool in future research will be dependent on the research questions.

The issue with the specificity of targeted PCR is that the results uncovered can only fall within the prior expectations of the researcher. Another issue with this degree of specificity is that the design of primers can only be based around the variable regions that have already been published and identified. As most reference genomes come from modern contexts, they may not be reflective of the variation of genomes in the past which may explain why the novel primers that were designed to amplify variable targets in Chapter 5, were unsuccessful. The results of this research suggests that a genetic approach that has the sensitivity of a targeted approach, but is also less discriminatory, might be the ideal for palaeoparasitology.

6.6 Limitations of the approach in this thesis

6.6.1 Problems with the genetics reference databases

Many of the problems detected during the genetic approaches taken in this thesis stem from the incomplete nature of the modern helminth genetic reference sequences available for comparison. This lack of reference genomes is incongruent with the number of people infected with helminths and the medical significance of these taxa. There are a small number of full genomes published so much of the database is built on modern studies that focused on short mitochondrial or RNA sequences and these studies tend to be small in scale.

This is complicated further by the mis-assignment of parasitic species in genetic databases (Valkiu et al., 2008). This could either occur due to mistakes made during morphological examination or by researchers relying on the host organism for identification rather than the parasite itself. Molecular evidence is increasingly suggesting that some of the past assumptions on parasite specificity were wrong (Poulin and Keeney, 2008), and therefore the misrepresentation of parasite taxa could be widespread. As well as taxonomic mis-assignment, there are also problems of genetic contamination in helminth genomes either stemming from the host, commensal parasites or from laboratory contamination (Coghlan et al., 2018). For example, during the initial data analysis steps in this thesis, PhiX was regularly identified amongst the helminth BLAST hits. PhiX is a quality and calibration control used during sequencing based on a well characterized bacteriophage and is a contaminant that also plagues microbial genome research (Mukherjee et al., 2015).

Accurate de novo construction of complete genomes with accurate taxonomic information is therefore vital to future research on these parasites. A team lead by Matthew Berriman at the Wellcome Sanger Institute have been running the 50 Helminths Project to address this lack of genomic data, however this project has been run concurrently to this DPhil. Whilst their work is published on Wormbase (http://parasite.wormbase.org/), many of the genomes are in contig form so are not in order, are not labelled and contain some contaminant sequence (PhiX, primers, and exogenous DNA).

There have been very few studies focused on sequencing entire genomes of helminths prior to this project due to the size and the complexity of the genomes in comparison to prokaryotes or protozoan pathogens, and the large effective population sizes making it difficult to distinguish between multiple loci and haplotypic variation (Wit and Gilleard, 2017). The mitogenomes are difficult to reconstruct as A T rich regions makes sequencing problematic and prone to errors, pseudogenes and gene rearrangement complicates sequence construction, and the low amounts of tissue

available from small worms results in pooling individuals causing excessive sequence heterogeneity (Jex et al., 2010).

In the case of nuclear genomes, many of the issues stem from the high presence of satellites. Satellites occur in the centromere region which in monocentric species, is one locus. Subirana and Messegeur (2013) found in a study of nematodes (which are usually holocentric) that their genomes contained frequent short motifs, either isolated throughout the genome or as long tandem repeats, which they suggested was due to the fact that the centromeric function is spread over the chromosome. All of these factors combined explain why the construction of helminth genomes has been historically slow.

6.6.2 Methodology developments for improving the reference database.

Currently the main platforms used for sequencing are Ion Torrent, Pacific Biosciences and Illumina which all fall under the classification of second generation sequencing platforms (Quail et al., 2012). Whilst these platforms have revolutionised genetic research, they are limited to producing reads between 35-700 bp. This is problematic for research aiming to construct helminth genomes as it is difficult to assemble these genomes when the length of the repeats are longer or equal to the length of the reads (Korhonen et al., 2016). Repeat regions therefore remain unresolved and as these are a large component of helminth genomes, this partially explains the incomplete nature of many of the reference sequences.

Fortunately, this issue may begin to be resolved with the rise of third generation sequencing. This includes single molecule real time sequencing (SMRT) by Pacific Biosciences and nanopore sequencing Oxford Nanopore's Technology. This type of sequencing is currently considered as costly and prone to error however, these problems will eventually diminish with further research and they have the key advantage in that they can produce longer reads (Schadt et al., 2010). The MinION sequencer has already been successfully applied to produce the genome of *Nippostrongylus brasiliensis* (Eccles et al., 2018). This paper suggests that over time, the reference database of helminth genomes will significantly improve as it will be easier to accurately detect satellites, and therefore assemble full genomes.

6.7 Next steps to develop genetic approaches to palaeoparasitology

6.7.1 Development of bioinformatic approaches

With an improvement to the modern reference sequences, the bioinformatic approaches to palaeoparasitology can evolve. Metagenomic studies are a growing field both in modern and ancient DNA studies with the goal of retrieving the genome sequences of a community or organisms inhabiting a common environment (Hugenholtz

and Tyson, 2008). In this thesis, BLAST alignments were run and then reads that were uniquely assigned to an individual species were visually assessed in MEGAN. Alternative approaches use alignments to clade specific marker genes (MetaPhlAn, Segata et al., 2012) or k-mers (Kraken, Wood and Salzberg, 2014) instead which makes the process faster and less computationally intense. These alternative approaches are often the basis of automated pipelines designed for metagenomic analysis.

These automated pipelines align reads to reference databases that include sequences of interest to produce statistical analyses and graphs visualising the taxonomic composition of the reads. For example: metaBIT utilises MetaPhlAn and automatically produces krona hierarchical pie charts, heatmaps and stacked bar charts so researcher can visually assess the composition of the reads, as well as running principle coordinates analyses, hierarchical clustering and, linear discriminant analysis (Louvel et al., 2016). Whilst many toolkits with embedded databases have been developed, they have been designed for the analysis of microbial communities and presume a modern context, therefore they lack the robust and stringent criteria required when working with short and damaged reads (Parducci et al., 2017), and they are not specialised for the detection of helminths.

A step that should be taken for the study of palaeoparasitology in the future is the development of an equivalent tool kit specifically for the identification of helminths, and possibly other pathogenic organisms that are not microbial, that could be found in latrines, coprolites and single burial samples. CLARK would be a good option for building this tool as it has high precision and speed, you can build a custom database to use for analysis and the reference sequences used for the database do not need to be complete genomes (Ounit et al., 2015). The quality of the results from a tool such as this however, is reliant on the construction of a complete and appropriate database (Willerslev et al., 2015).

As mentioned in the Supplementary of Chapter 5 (5.10.3), when a BLAST run was conducted with just helminth genomes there was a number of positive hits that were later identified to be more strongly associated with other taxa. This indicates that either the database would require carefully considered sequences to represent outgroups or, conserved stringency measures to ensure that only the most robust matches were retained. To develop, test and perfect an appropriate reference database and establish the right parameters for the use of a CLARK based pipeline, would take a significant amount of research. Ideally this would be developed and used in conjunction with other bioinformatic tools for the visualisation and statistical analysis of the taxonomic

composition of shotgun data. This work was beyond the scope of this thesis but would be an invaluable addition to genetic approaches in palaeoparasitology.

6.7.2 Enrichment Strategy

As well as exploring alternative bioinformatic approaches to palaeoparasitology, future research could also explore alternative genetic extraction and amplification approaches. There is a technique that has the capacity to enrich for species and gene regions of interested but, is less selective and restrictive than using single target specific primers. It is called Hybridization-Based Target enrichment, also known as baiting. In this approach endogenous DNA attaches to target specific probes which can then be separated prior to sequencing from unbound (exogenous) DNA either in solution or on a solid support-like microarray (Mamanova et al., 2010).

Baiting approaches are often utilised for research aiming to extract and amplify DNA from a mixed context, be it separating parasite DNA from the host (Geniez et al., 2012; Guo et al., 2015) or degraded endogenous DNA, found in environmental or ancient samples, from contaminant DNA (Rizzi et al., 2012). Enrichment strategies have the capacity to retrieve full genomes from ancient samples (Carpenter et al., 2013). The approach was even successfully applied for the reconstruction of mammoth genomes despite the lack of a modern reference. Instead Enk et al. (2014) used the most closely related living relative, the African elephant (*Loxodonta africana*), as the foundation for the bait design.

Another strength of baiting is that it can also be used to identify multiple taxa of interest, which is a particularly useful trait for palaeoparasitology. There is a balance to be struck however, with the number of species and the number of loci that can be identified as the cost scales with the number of different baits within a set. Whilst baits can enrich for off-target species, unsurprisingly the capacity to do this decreases with increasing sequence divergence (Paijmans et al., 2016). There are a number of vendors who can produce custom baits including: Agilent (SureSelect product), NimbleGen (SeqCap EZ product), Flexgen and MYcroArray (Mertes et al., 2011) but it is down to the researcher to design a good bait set.

The capacity to build a good bait set successfully is dependent on the complexity of the genomes of interest and the availability of good genetic resources as the basis for the design (Heyduk et al., 2016). Currently the most complete reference genomes available for multiple species of helminth is the mitogenome, so these would be the clear regions to use for bait design. As the targets would be based on modern reference

sequences, it limits the ability for researchers to identify novel genomic regions (an issue noted by Bos et al., 2011). This approach would however, have the capacity to be able to rapidly recover reads from multiple helminth species and gene regions, and also recover a high proportion of endogenous reads for each sequencing run. Future research should focus on developing this approach as it has the potential breadth and depth to make it a cost-effective way to assess the spread and population dynamics of multiple helminths, rather than restricting analysis to one species at a time.

6.8 Is the impact of helminths always negative?

The motivation in this thesis for exploring novel microscopic and genetic approaches in palaeoparasitology was to improve the capacity to study the epidemiology of helminths over time, rather than focusing on their significance for inferring diet and hygiene practices. As helminths are still a significant health problem, historical case examples on the spread and resilience of helminths have the potential to be used to inform the decisions made by modern medical and policy practitioners. There are established problems of reinfection in communities following chemotherapy treatment (Jia et al., 2012) so some researchers argue that the ultimate goal should be to eradicate these organisms (Truscott et al., 2014).

In this thesis we use European sites to explore how these organisms became eradicated and to assess which cultural and environmental changes had the biggest impact on helminth prevalence without the use of wide-scale and systematic chemotherapy. Tracing population dynamics of helminths using genetics has the capacity to add to this narrative as it would provide complimentary data to distinguish cases of population growth and decline, and whether this was impacted by an influx of infected individuals from another region. Whilst most people would agree that the removal of pathogenic organisms is a positive goal, there are some counter points to be considered.

This thesis highlights the utility of using parasites as a proxy for understanding human behaviour. Whilst our focus is on past societies, the ability for helminth population genetic patterns to illustrate hosts evolutionary and demographic history is still applicable for modern populations (Whiteman and Parker, 2005). This benefit is usually discussed by conservationists in relation to the maintenance of animal parasites and they also highlight the importance of maintaining biodiversity (Dougherty et al., 2015). As a general rule however, human health takes precedence over maintaining biodiversity. A more convincing argument for the preservation of pathogenic organisms is that they have the capacity to be used for medical research, beyond the development

of direct treatments. For example: there are calls to eradicate the schistosomes but these species can be used as model organisms for the study of stem cell biology, acute and chronic inflammation, liver and gut fibrosis, and T helper 2-type responses in humans as they are long lived, have strong immunogenic properties and cause inflammation in their hosts (Hsieh and Mentink-Kane, 2016). Some pathogenic organisms can be maintained in laboratory settings which would negate the need to put human hosts at risk.

Finally, whilst parasitic infections are generally detrimental, these organisms can become allies either by performing a function the host cannot perform alone or by modifying a life history trait which is beneficial in certain environments (Fellous and Salvaudon, 2009). The association between the reduction of helminths and the rise of autoimmune responses first began to be explored in the early 1970s (for example Bazaral et al., 1973), more colloquially known as the hygiene hypothesis. This association is allowing researchers to explore the causes of allergies which could eventually be used to develop drugs for the treatment or prevention of allergic diseases (Flohr et al., 2008). In controlled cases where an individual is suffering from extreme allergic responses, infection with helminths can be classified as a treatment (Reddy and Fried, 2007) however, whether this benefit outweighs the negative impacts of helminth infection in the broader population is up for debate.

Whilst this thesis has contextualised helminth infections as a negative, a flip in the perception of these pathogenic organisms highlights alternative ways to study them in archaeological contexts in the future. Allergies cannot currently be reliably traced in the archaeological record however, according to the hygiene hypothesis we should expect an inverse relationship of the prevalence of helminths to the prevalence of autoimmune diseases. To establish whether this association can be applied broadly in the archaeological record, a study of helminths from contexts where there are also historical records of the prevalence of autoimmune diseases would need to be established. This is one further example of how helminths in archaeological sites can be studied to understand health of past populations, so it seems likely that this thesis has only scratched the surface of the utility of the study of palaeoparasitology.

6.9 Summary of the key next stages for research

- Future research should focus on single burial analysis to grow the database of historical prevalence rates that has begun to be developed in this thesis.
- A data sharing platform of microscopic count data should be developed as it would improve researchers' capacity to address questions limited by the small size of subgroups that can occur when analysing single burial prevalence rates.

- Questions that could be addressed once there has been an increase in the number of single burials analysed include: association of helminths and osteological indicators of stress, helminth prevalence in association with rural and urban sites, the effects of specific WASH behaviours on helminth prevalence, distinguishing between site specific differences and those that reflect broader temporal changes and finally, why there is a difference in the dominant helminth uncovered from single graves versus communal contexts.
- For genetic analysis of museum samples to be feasible, research should be undertaken to identify samples with clear details of their curative history so only the samples with the highest probability of being successful are damaged.
- Both targeted PCR and shotgunning approaches should be used to assess helminths in communal and single grave samples, but future research should decide which method to utilise based on their research question.
- The development of an automated pipeline that uses a conserved approach to compare shotgun reads to a curated database of helminth genomes is a clear next step to improve the utility of shotgunning for helminth analysis in archaeological contexts.
- Enrichment is another approach that may improve the proportion of endogenous reads to environmental contaminant, so it is a technique to be explored when working with these difficult sample types.
- A research question that has not been addressed in this thesis is the association of helminth prevalence and the rise of allergies. Future research based on archaeological and historical evidence is one approach that could be used to interpret the relationship between these two pathologies.

6.10 Concluding remarks

The goal for this thesis was to explore epidemiological approaches to the study of helminths in archaeological sites. Microscopic analysis of sediment samples from the pelvis of individual burials is a robust approach for exploring prevalence rates and risk factors in the past and this thesis undertook the largest study of these sample types to date. We also established that age, sex and population density were risk factors that could impact the prevalence of helminths in historical populations. Parallels can be drawn between historical and modern prevalence rates which was used to: (i) develop a baseline infection rate that could be used as a proxy to analyse the effectiveness of interventions to counter infection; and (ii) identify that hygiene practices are sufficient to substantially deplete the prevalence of helminths without the use of chemotherapy

however; the effectiveness of hygiene practices depends on the type and breadth of interventions utilised.

The genetic research developed here highlights novel approaches to helminth species identification both in museum samples and sedimentary contexts, whilst also bringing to light potential steps that could be taken to allow more effective research to be undertaken in this area. As it stands, phylogeographic analysis in palaeoparasitology remains limited, however when improvements are made to the reference database I am confident that many of the issues encountered in this thesis will be overcome. The approaches developed here provide a helpful foundation to develop a methodology in palaeoparasitology that can be used to produce robust conclusions which are specific and quantifiable on the risk, prevalence and spread of helminths in historical populations. Regardless of whether the elimination of helminths should be the ultimate goal in medical practices, the capacity to make these specific conclusions on past societies is vital if the work is to be of use for informing modern practices.

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Chapter Seven: Appendix

In the course of this DPhil, I have contributed to the academic environment of my research group whilst maintaining my own independent research. Whilst I was not the lead for all of these project, I have included them to illustrate my academic development.

They Include:
7.1 Report on extraction of cattle bones.

7.2 Microscopy reports for sites where samples were analysed by microscopy.

7.3 Allen, R, Ryan, H, Davis, B W, King, C, Frantz, L, Barnett, R, Linderholm, A, Loog, L, Haile, J, Lebrasseur, O, White, M, Kitchener, A, Murphy, W and Larson, G (In Prep) "Mitochondrial divergence predicts biological feasibility of archaic human hybrids."

I have also published work with my colleagues including:

Lebrasseur, O., Ryan, H. & Abbona, C., 2018. Bridging Archaeology and Genetics. In E. Piskin, A. Marciniak, & M. Bartkowiak, eds. Environmental Archaeology. Springer, pp. 111–132.

Irving-Pease, E.K. et al., 2018. Paleogenomics of Animal Domestication. In C. Lindqvist & O. . Rajora, eds. Paleogenomics, Population Genomics. Springer, pp. 1–48. Available at: https://doi.org/10.1007/13836_2018_55.

Cattle Report

Bones were extracted based on Dabney (2013) and Damgaard (2015) and then libraries were built based on the protocol laid out by Meyer (2010). Samples were analysed using the tape station to preliminarily assess the quantity of endogenous DNA and then samples were pooled and cleaned based on this concentration. After size selecting for fragments between 150-300 base pairs long, samples were sent to Copenhagen for Hi-Seq sequencing. Below are the results from the first run.

Extract No.	Sample name	Dating (14C)	Mapped Reads	% Mapped-Q30	MT_reads	Duplicates
CC1	23127	2902 +-21	98	0.07545272	0	0.3571
CC2	23128	2952 +-22	4500	2.7121378	0	0.4236
CC3	23129	2949 +-20	240	0.2589332	0	0.4167
CC4	23130	2906 +-22	349	0.32352765	0	0.447
CC5	23131	2921 +-22	923	0.13553196	0	0.7335
CC6	23134	6962 +-26	214	0.00657505	0	0.5888
CC7	23139	7445 +-29	2552	0.03995458	0	0.8088

Of all the samples, only CC2 (23128) could possibly be used for further investigation as the % Mapped Q30 value is over 1%. However the lack of mitochondrial reads factor and the high clonality may be limiting factors. To try and improve upon these results, the process above was repeated but with the addition of a bleaching step which can in some cases increase the recovery rate of endogenous DNA recovery. After grinding the bone, 0.2% bleach was added and agitated for half an hour at room temperature before being washed through with distilled water 3 times. Below are the results of this run. A repeat of 23131 could not be undertaken as this bone had been used up in the previous run.

Extract No.	Sample name	Dating (14C)	Mapped Reads	% Mapped-Q30	MT_reads	Duplicates
CC9	23127	2902 +-21	24414	0.26066403	3	0.7582
CC10	23128	2952 +-22	12123	0.19849492	3	0.4644
CC11	23129	2949 +-20	23130	0.27085229	8	0.5303
CC12	23130	2906 +-22	10992	0.13261903	0	0.845

CC13	23134	6962 +-26	41149	0.42320357	0	0.925
CC14	23139	7445 +-29	20010	0.07796336	1	0.7778

Whilst the bleaching step improved the recovery of mitochondrial DNA, it had inconsistent affects on the total recovery of DNA sequences (% Mapped-Q30). Regardless of whether the bleaching step improved or decreased the % of the genome recovered, none of the samples produced enough reads for meaningful analysis to be undertaken.

Parasitological analysis of the samples from County Hotel, Dover.

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

Seven soil samples were collected from early, post-Medieval cess tanks and analysed by microscopy. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

Four of six samples contained at least one putative egg. *Trichuris* sp. was the most common helminth (parasitic worm) identified in four samples (57.14% prevalence). Other eggs identified were *Ascaris* sp. (2 samples) and, an unidentified egg (see Figures 1-3 for microscopy examples). The nematodes (Roundworm) *Ascaris* sp. and *Trichuris* sp. are transmitted via a faecal-oral route and are the most common helminths found in archaeological samples. Today both genera are prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, they are almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel). The number of *Ascaris sp.* and *Trichuris* sp. have been summarised in Table 1. Generally, eggs identified by microscopy are seen as a minimum estimate for the prevalence of helminths in the living population.

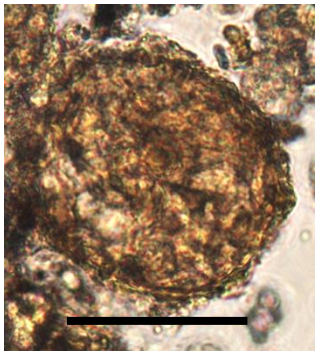


Figure 1: *Ascaris* sp. identified in sample 8064-10 (scale bar represents 50 µm).

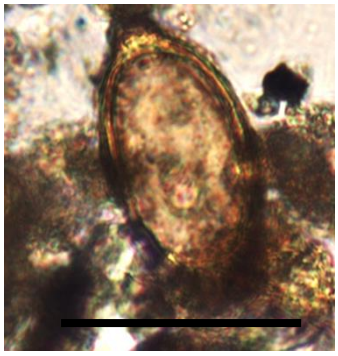


Figure 2: *Trichuris* sp. identified in sample 8030 4-2/3 (scale bar represents 50 µm).

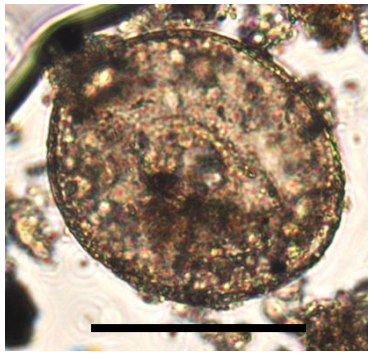


Figure 3: Putative parasite egg identified in sample 8030 4-1/3 (scale bar represents 50 µm).

Sample ID	*Ascaris* sp. [per g]	*Trichuris* sp. [per g]
8030-4 (1/3)	0	164.74
8030-4 (2/3)	0	85.47
8030-4 (3/3)	0	0
8064-10	269.78	89.93
8064-12	279.33	186.22
8074-9	0	0
8203-41	0	0

Table 1: Number of parasite eggs per gram soil sample. The estimates are based on the mean number of parasite eggs counted during microscopy.

Parasitological analysis of the samples from Russel Street, Dover.

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

Thirteen soil samples were collected from Medieval/Post Medieval cess tanks and garderobe shafts and were analysed by microscopy. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

Seven of thirteen samples contained at least one putative egg. *Ascaris* sp. was the most common helminth (parasitic worm) identified in five samples (38.46% prevalence) however they were found at a very low abundance (usually only one egg identified). Other eggs identified were *Trichuris* sp. (2 samples), a potential *Taenia* sp. egg and, a putative unidentified helminth egg (see Figures 1-3 for microscopy examples). The nematodes (Roundworm) *Ascaris* sp. and *Trichuris* sp. are transmitted via a faecal-oral route and are the most common helminths found in archaeological samples. Today both genera are prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, they are almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel). The number of *Ascaris sp.* and *Trichuris* sp. have been summarised in Table 1. The cestode (Tapeworm) *Taenia* sp. are associated with consumption of raw, undercooked or under cured red meat. Generally, eggs identified by microscopy are seen as a minimum estimate for the prevalence of helminths in the living population.



Figure 1: *Trichuris* sp. identified in sample 7147 (scale bar represents 50 µm).

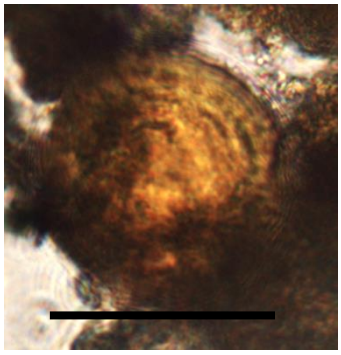


Figure 2: Putative *Taenia* sp. identified in sample 6853 (scale bar represents 50 µm).

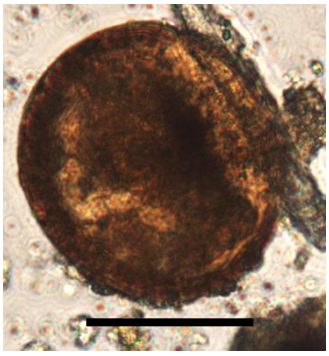


Figure 3: Unidentified helminth in sample 7111 (scale bar represents 50 µm).

Sample ID	*Ascaris* sp. [per g]	*Trichuris* sp. [per g]
6768-78	0	74.29
6768-72	73.10	0
6769-79	0	0
6772-89	0	0
6773-85	99.21	0
6790a-90	0	0
6798-84	0	0
6853-104	0	0
6857-119	0	0
7111-140	82.10	0
7116-142	181.82	0
7147-147	0	78.86
7148-148	88.18	0

Table 1: Number of parasite eggs per gram soil sample. The estimates are based on the mean number of parasite eggs counted during microscopy.

NB: Due to technical issues, there are no photographic records of *Ascaris* from this site.

Parasitological analysis of the samples from Peugeot Garage, Canterbury.

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

Eighty soil samples were collected and analysed by microscopy. All soil samples were collected from the pelvic region of skeletons and are believed to be Roman in age. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

Thirty one out of eighty (38.75%) of the pelvic-soil samples contained at least one identifiable egg. The most common helminth (parasitic worm), found within 27 individuals, was *Ascaris* sp.. Other eggs identified included *Diphyllobothrium latum* (1 sample), putative *Taenia* sp. eggs (3 sample), and putative unidentified helminth eggs (2 sample) (see Figures 1-3 for examples). To see which skeletal samples had identifiable eggs within the soil of their pelvic region, see Table 1. The nematode (Roundworm) *Ascaris* sp. is transmitted via a faecal-oral route and is one of the most common found in archaeological samples. Today *Ascaris* sp. is prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, it is almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel). *Trichuris* sp. are regularly found to co-infect in regions where *Ascaris* sp. are prevalent so it is a surprise that no *Trichuris* sp. was found in these samples. The cestode (Tapeworm) *Taenia* sp. are associated with consumption of raw, undercooked or under cured red meat whilst *Diphyllobothrium* sp. are associated with the consumption of raw, undercooked or under cured fresh-water fish. The prevalence rates seen in the Peugeot Garage, Canterbury, are comparable to many other archaeological sites, see Figure 4 for a summary of the results.

Prevalence rates were also assessed against skeletal traits provided. In modern populations, children are more likely to be infected with parasites transmitted by the fecal-oral route than adults however, in the samples from Peugeot Garage there did not seem to be a significant difference between age group and prevalence rate (see Figure 5). In regards to sex, seven out of fifteen (46.67%) of females suffered from helminth infections whilst only nine out of twenty-six males were infected (34.62%). Considering the overall low prevalence rates, it is difficult to say whether the patterns detected reflect sampling bias or the living infection loads.

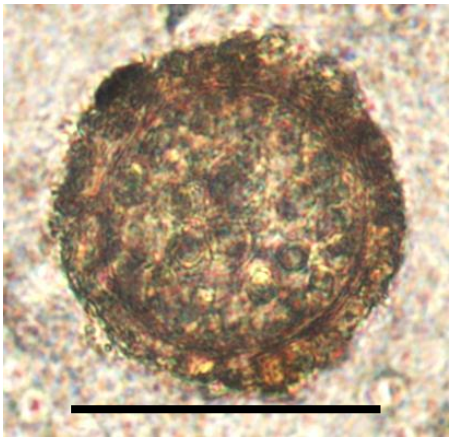


Figure 1: *Ascaris* sp. identified in sample 3931 (scale bar represents 50 μm).

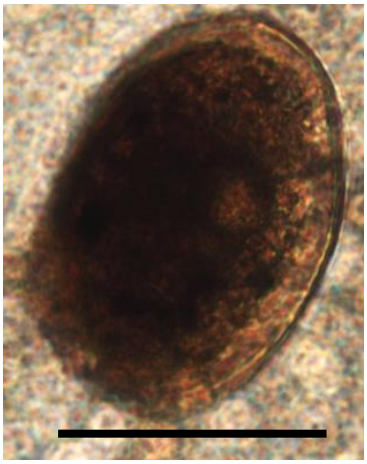


Figure 2: *Diphyllobothrium latum* identified in sample 2588 (scale bar represents 50 μm).

ID Number	*Ascaris*	*Trichuris*	*Taenia*	*Diphyllobothrium*	Unidentified Egg	Total
3682	1					1
3568						0
2579						0
3402	1					1
3743	1					1
3474						0
3135						0
3658	1					1
3931	2					2
5079	1					1
3674	1					1
3467						0
3490	1					1
3921						0
3529	1					1
3448	2					2
3698						0
3644	1					1
3390	1					1
3978	1					1
3360						0
3752						0
5167	1					1
3803						0
3755	1					1

3385						0
2524	2					2
3680						0
3944						0
3690						0
3713						0
3571						0
3603						0
2588			1	2		3
3611	1					2
3544						0
3616						0
3686	1					1
3867						0
<389>						0
3994						0
3364						0
3425						0
3271						0
3275	1					1
<376>	1					1
No label 1						0
3550						0
3154						0
5193						0
5237						0
3638						0
3370						0
3410	1					1
3576						0
3818						0
3830						0
3771						0
3591						0
3695	1					1
3737						0
3614						0
3480	3					3
3753	1					1
3065	1					1
3101						0
3502	1		1			2

3080	2					2
3104						0
2565						0
3432			1			1
3972						0
3576						0
3394						0
3541						0
3835	1					1
3625					1	1
No label 2						0
3463						0
3413						0

Table 1: The total number of eggs identified per sample after two rounds of microscopy.

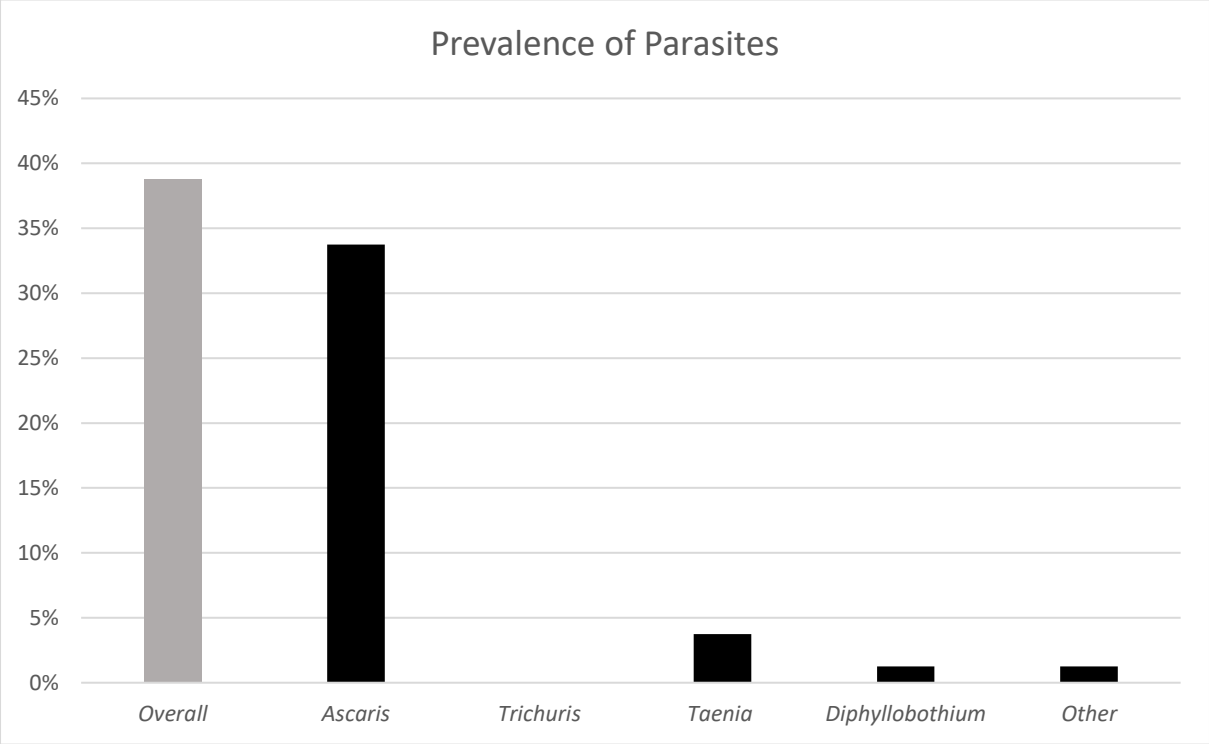


Figure 4: Overview of the prevalence of parasites.

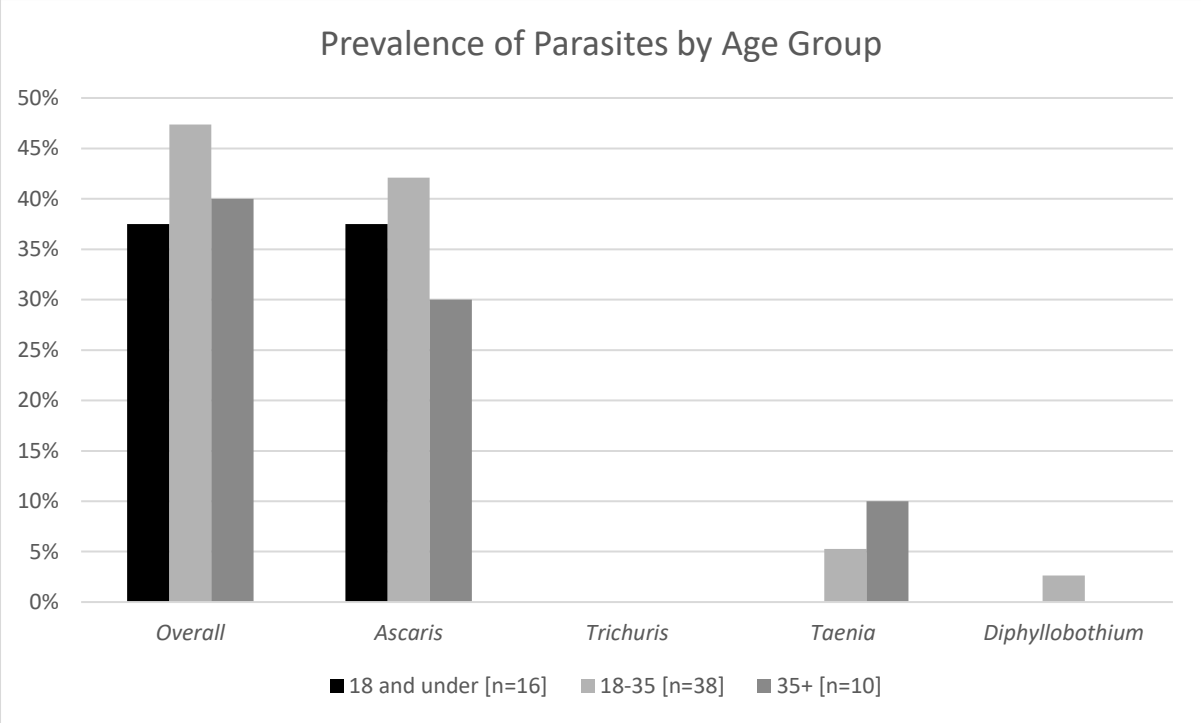


Figure 5: Overview of the prevalence of parasites by age group.

Parasitological analysis of the samples from Stoke Quay, Ipswich

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

28 soil samples were collected and analysed by microscopy. All soil samples were collected from the pelvic region of skeletons. Skeletal samples came from 3 different time periods: 5 samples date to 9-10th C, 11 date between 11-12th C and, 11 date between 12-15th C. For 1 sample, a date couldn't be identified (skeletal sample number 5969 and soil sample 5217). These samples were selected to coincide with the isotopic analysis undertaken by Eleanor Farber. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results and Interpretation

Twelve of twenty-eight (42.86%) of the pelvic-soil samples contained at least one identifiable egg. The most common parasite, found within 11 individuals, was *Ascaris* sp.. Other eggs identified included *Diphyllobothrium latum* (1 sample), a putative *Taenia* sp. egg (1 sample), and an unidentified egg (1 sample) (see Figures 1-2 for examples). A full description of which skeletal samples had identifiable eggs within the soil of their pelvic region is provided (Table 1). The nematode *Ascaris* sp. is transmitted via a faecal-oral route and is one of the most common found in archaeological samples. Today *Ascaris* sp. is prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, it is almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel). *Trichuris* sp. are regularly found to co-infect in regions where *Ascaris* sp. are prevalent so it is a surprise that no *Trichuris* sp. was found in these samples. The cestode *Taenia* sp. are associated with consumption of raw, undercooked or under cured red meat whilst *Diphyllobothrium latum* are associated with the consumption of raw, undercooked or under cured fresh-water fish. Two individuals suffered from co-infection, all individuals had 2 different identifiable parasites. Generally, eggs identified by microscopy are seen as a minimum estimate for the prevalence of helminths in the living population. The prevalence rates seen in the Stoke Quay, Ipswich are comparable to many other archaeological sites, if anything on the higher end of the incidence range, see Figure 3 for a summary of the results.

Prevalence rates were also assessed against skeletal traits plus the isotopic results and, some trends could be found within the data. Age of skeleton seemed to be a predictor of infection with younger individuals being more likely to host a parasite of any kind (see Figure 4). This reflects the pattern of infection in modern populations but, only three of the archaeological individuals were identified as under 18. Through nitrogen analysis of bone collagen, 16 individuals have been identified as having relatively high trophic level diets (above 13‰). These individuals were less likely to be infected with parasites, in particular *Ascaris* spp. (see Figure 5). In addition, the three individuals identified by Eleanor Farber as non-local from Strontium and Oxygen isotopic analysis of tooth enamel (20288, 20082, 20861) all have parasite eggs present. With genetic analysis, we may be able to identify whether these parasites have a unique haplotype that would provide further support to their identification of being non-local individuals.

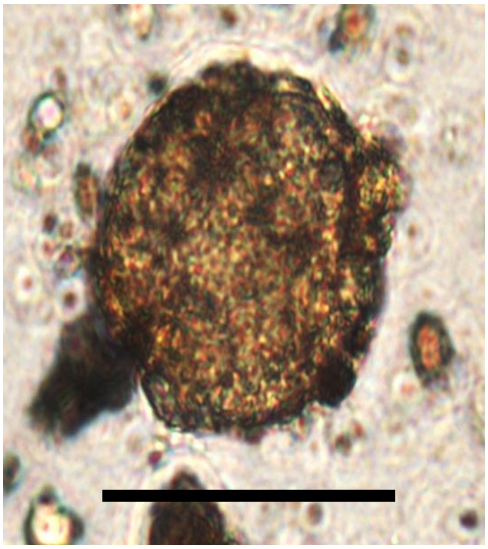


Figure 1: *Ascaris* sp. identified in sample 20255 (scale bars represent 50 μm).



Figure 2: *An unidentified egg* in sample 20402 (scale bars represent 50 μm).

Skeletal number	ID Number	Ascaris	Trichuris	Taenia	Diphyllobothrium	Unidentified Egg	Total
5699	5166	3					3
5968	5216	1					1
5969	5217						0
6249	5257						0
6907	5338						0
7268	5584						0
7599	5630						0
8517	5829	1					1
8532	5804						0
8866	5863						0
20060	20014						0
20292	20082	1		1			2
20348	20105	1					1
20580	20193						0
20633	20230						0
20748	20228	2					2
20801	20255	2					2
21032	20305						0
21348	20402	2				1	3
21686	20505						0
21699	20508						0
22114	20639	1					1

22272	20677						0
22527	20781						0
22785	20859						0
22989	20861	1					1
23017	20880				1		1
23141	20909	1					1

Table 1: The total number of eggs identified per sample after two rounds of microscopy.

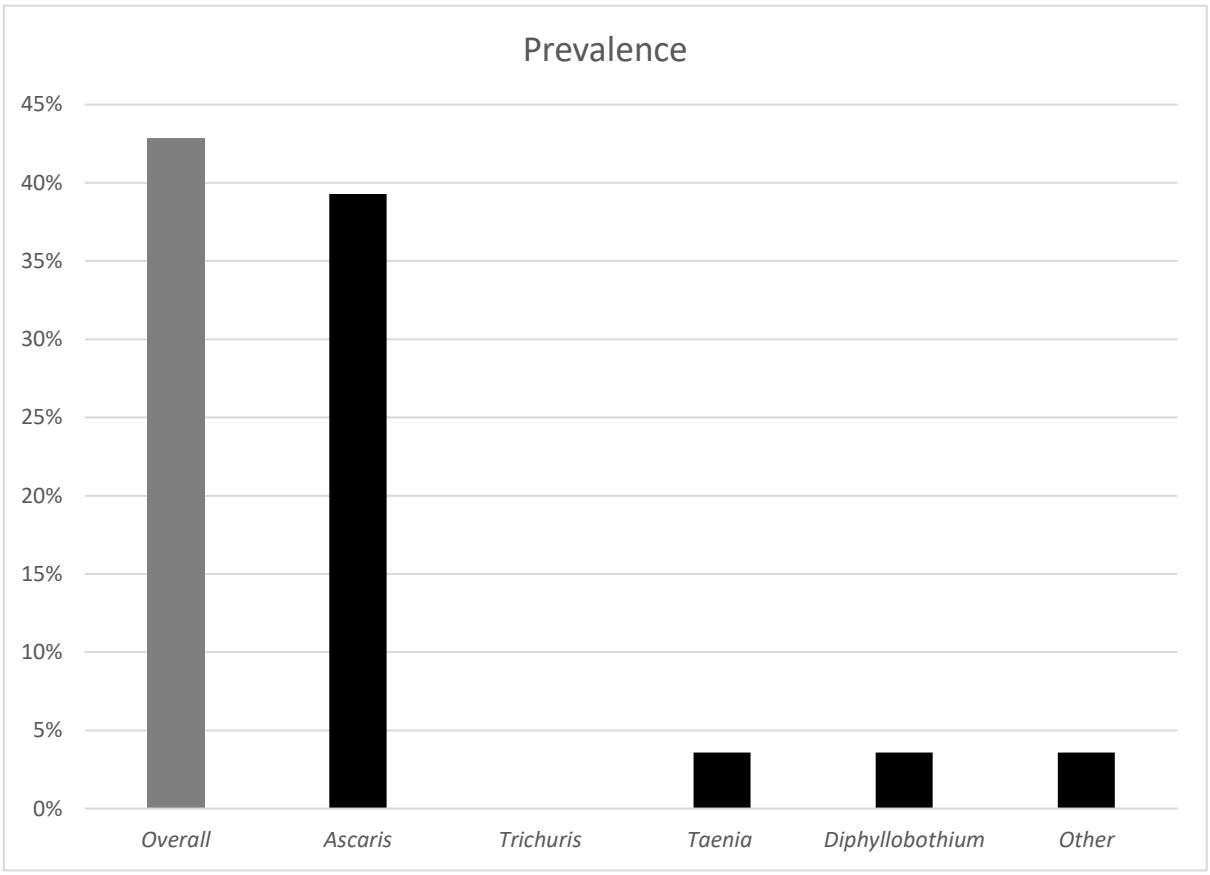


Figure 3: Overview of the prevalence of parasites.

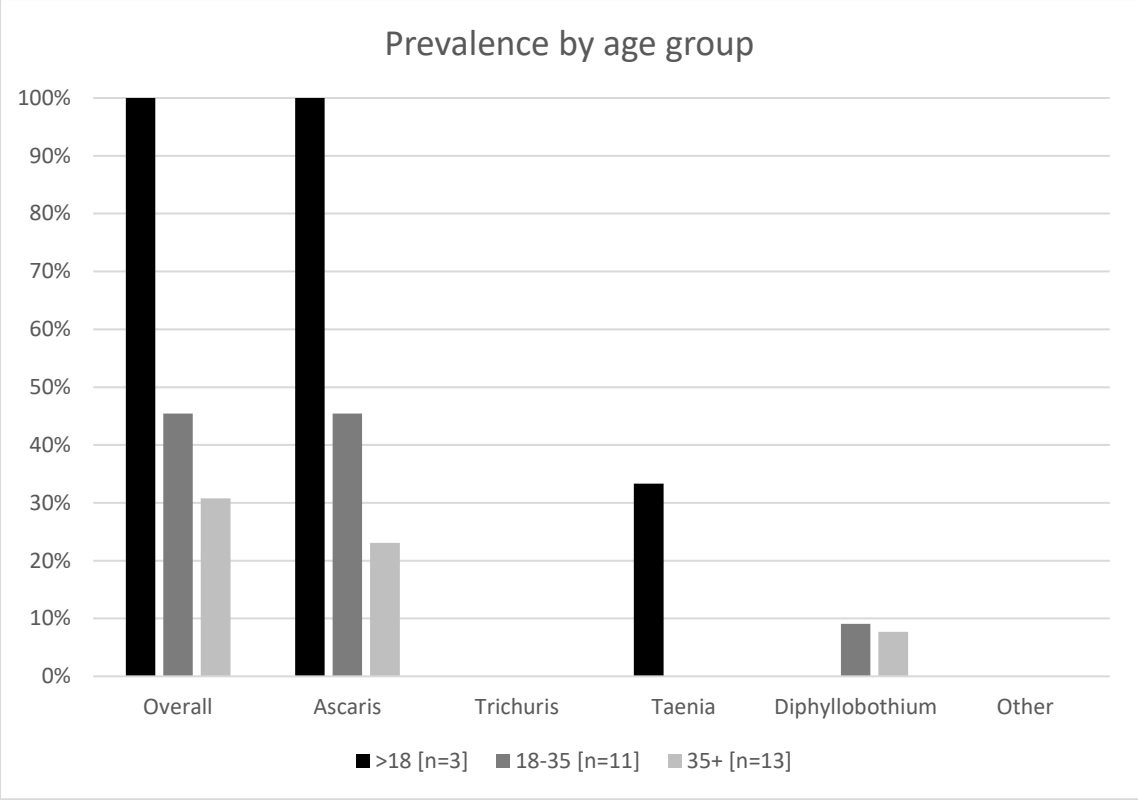


Figure 4: Overview of the prevalence of parasites by age group.

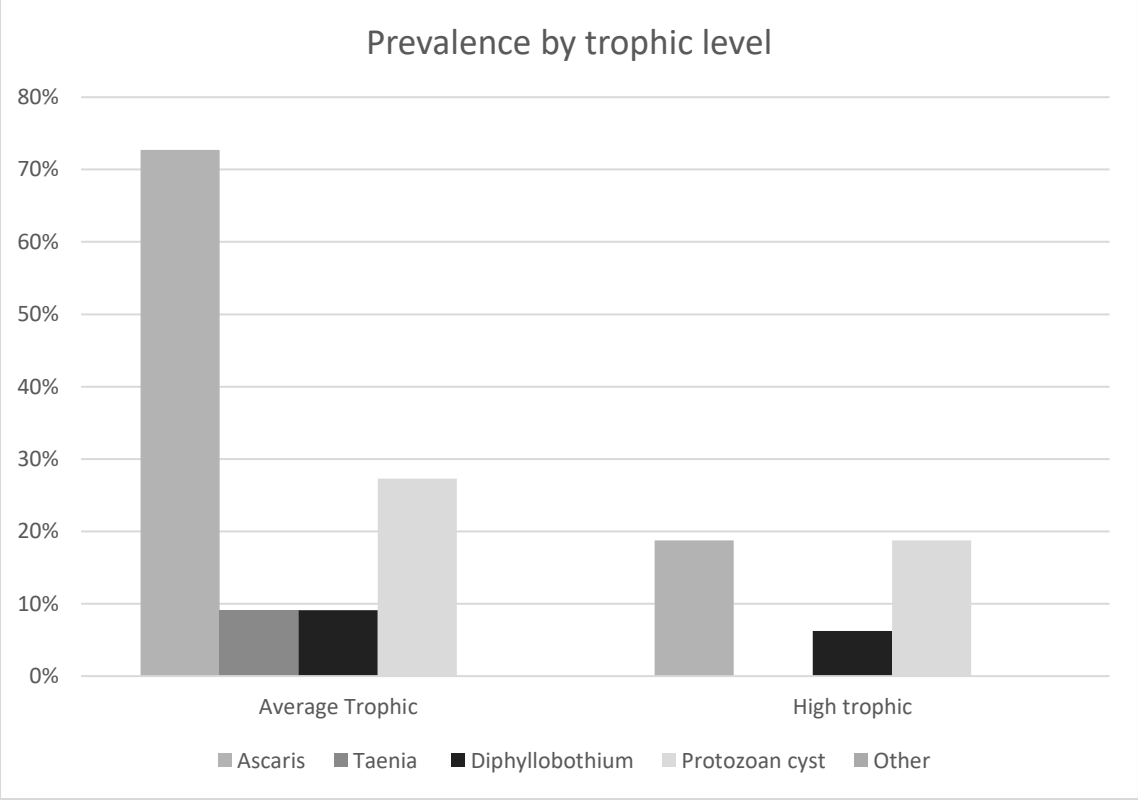


Figure 5: Overview of the prevalence of parasites by trophic level as identified by Eleanor Farber.

Parasitological analysis of the samples from Vilhelm Werners Plads, Odense (OBM 9776).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

Ten soil samples were collected from a range of contexts (human and animal) and analysed by microscopy. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

All ten samples contained helminth eggs. The most common helminths, *Ascaris* sp. and *Trichuris* sp. were abundant in all samples except sample X1910 which had no detectable *Trichuris* sp.. The parasite densities of these genera can be seen in Table 1. It is worth noting that sample X2172 has particularly high levels of *Trichuris* sp. present. More generally, *Trichuris* sp. eggs were found in higher numbers than *Ascaris* sp.. The nematodes *Ascaris* sp. and *Trichuris* sp. are transmitted via a faecal-oral route and are the most common helminths found in archaeological samples. Each of these genera are known to have a number of species, which are host specific, that infect domestic or wild animals. The human specific species are prevalent in many parts of the developing world today particularly those with issues around sanitation infrastructure however, they are almost completely absent from modern Europe. To identify whether the eggs identified are human or animal specific would require genetic analysis as they are similar in shape and size to the human infecting species. For example, there is a lot of similarity between *A. lumbricoides* and *A. suis* (which infect both human and pigs respectively) whereas *T. trichuria, T. muris, T.suis* and *T.vulpis* are all similar sizes (infecting humans, mice, pigs and canines). In addition, a number of *Taenia* sp. were identified (in samples 251, 1164, 2172, 1896 and 4177) which are associated with the consumption of raw, undercooked or under cured red meat in humans. Interestingly, whilst *Ascaris* sp. and *Trichuris* sp. were found in the construction layers, no *Taenia.* sp were identified in these samples. Whilst putative helminths were identified in several samples, they could not be confidently identified to the genera level. Generally, eggs identified by microscopy are seen as a minimum estimate for the prevalence of helminths in the living population.

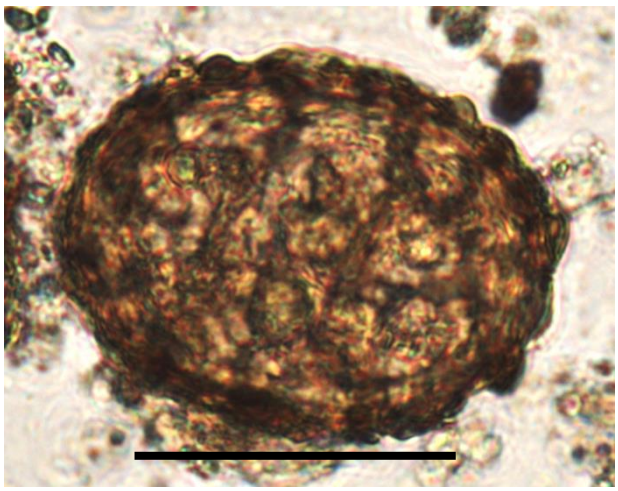


Figure 1: *Ascaris* sp. identified in sample 2172 (scale bars represent 50 μm).



Figure 2: *Trichuris* sp. identified in sample 1164 (scale bars represent 50 μm).

Sample ID	**Sample Context**	***Ascaris* sp. [per g]**	***Trichuris* sp. [per g]**
X251	Latrines	266.43	1154.53
X1164	Latrines	971.73	2031.80
X2172	Latrines	687.62	11886.05
X5422	Latrines	900	3200
X1896	Dung Layers	432.90	144.30
X1910	Dung Layers	216.76	0
X4177	Dung Layers	834.72	4340.57
X1702	Pit	1071.43	267.86
X2358	Construction Layer	278.81	929.37
X1036	Stable	366.97	275.23

Table 1: Number of parasite eggs per gram soil sample. The estimates are based on the mean number of parasite eggs counted during microscopy.

Parasitological analysis of the samples from Park Street, Birmingham (1N17PSTHL)

Report prepared by Hannah Ryan, University of Oxford, 2018 reviewed by Patrik Flammer and Adrian Smith.

Methodology

From the preliminary assessment of the site, 30 soil samples were collected and analysed by microscopy. All soil samples were collected from the pelvic region of skeletons. Skeletal samples came from 4 different trenches: 7 samples were from trench three, 5 from trench four, 13 from trench five and 5 from trench six. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed. Due to the low prevalence of eggs within single grave samples, 'eggs per gram' density was not calculated as the margin of error would be too high for these calculations to be meaningful.

Results and Interpretation

Four of thirty (13.33%) of the pelvic-soil samples contained at least one putative egg. Eggs identified include *Trichuris sp.* (1 sample), a potential *Ascarid* specimen (1 sample) and two potential eggs that are unidentifiable due to soil adhesion (see Figures 1-2 for examples). To see which skeletal samples had identifiable eggs within the soil of their pelvic region, see Table 1. The nematode *Trichuris* sp. is transmitted via a faecal-oral route and is one of the most common found in archaeological samples. Today *Trichuris* sp. is prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, it is almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel). None of the individuals suffered from co-infection. All of the individuals with putative eggs identified were adults but there was no identifiable correlation with either gender or the presence of pathology. Generally, eggs identified by microscopy are seen as a minimum estimate for the prevalence of helminths in the living population. The prevalence rates seen in Park Street, Birmingham are at the lower end of the spectrum for prevalence however, the values are still high enough that a population of helminths could be maintained. See Figure 3 for a summary of the results.

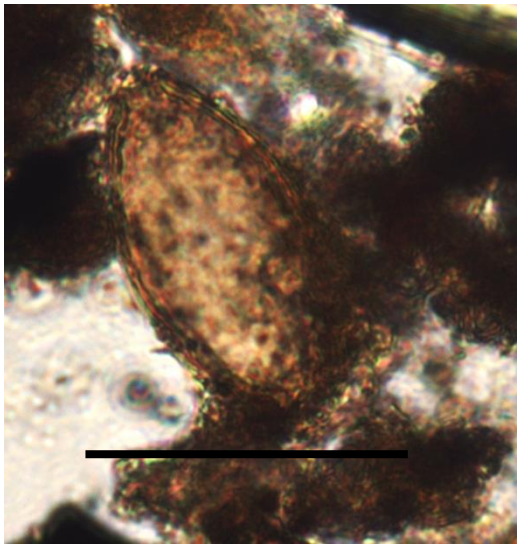


Figure 1: *Trichuris* sp. identified in sample 5122 (scale bars represent 50 μm).

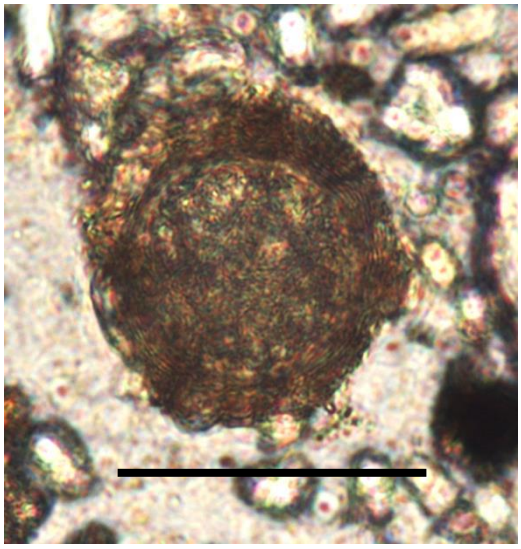


Figure 2: Potential *Ascarid* specimen identified in sample 3045 (scale bars represent 50 μm).

ID Number	Ascaris	Trichuris	Taenia	Diphyllobothrium	Egg (id in notes)	Total	Comments
3040						0	
3045					1	1	Ascarid
4017						0	
4028						0	
4066						0	
4089						0	
4093						0	
5036						0	
5038						0	
5040						0	
5064					1	1	Half covered in soil
5068						0	
5073						0	
5083						0	
5093						0	
6016						0	
6019						0	
6023						0	
6025						0	
6030						0	
3061						0	
3077						0	
3095						0	

3098						0	
3110					1	1	Half covered in soil
5097						0	
5107						0	
5110						0	
5122		1				1	
5127						0	

Table 1: The total number of eggs identified per sample after two rounds of microscopy.

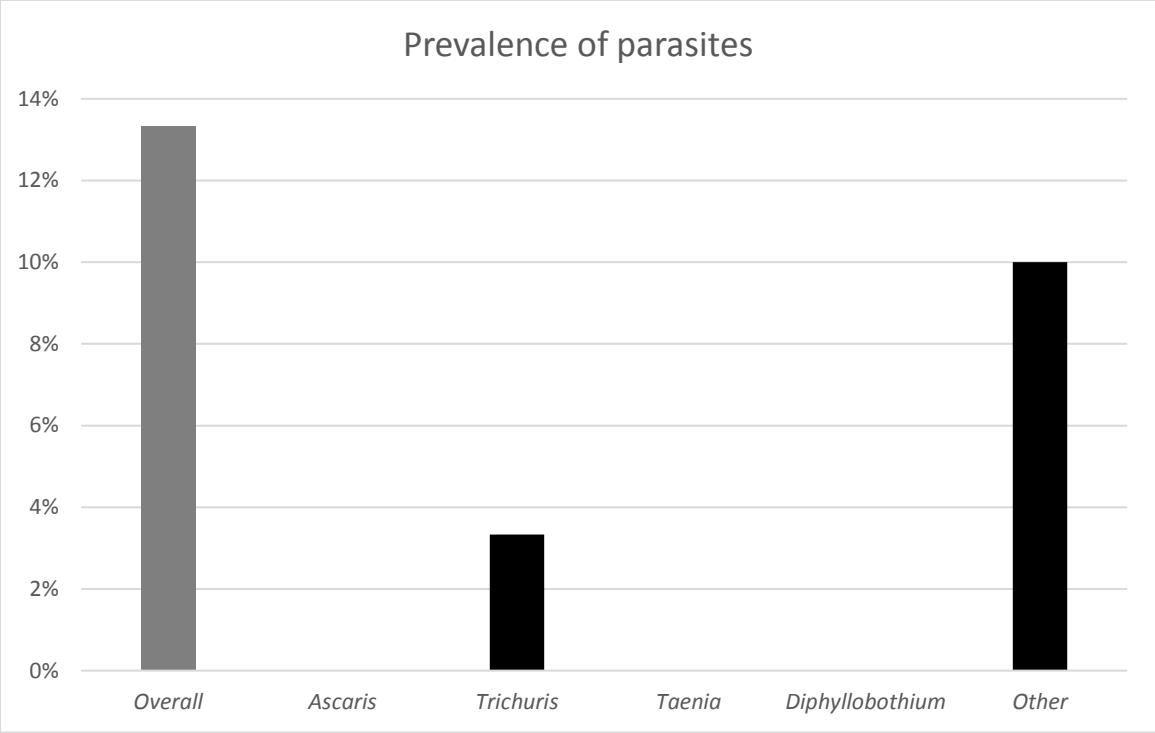


Figure 3: Overview of the prevalence of parasites.

Parasitological analysis of the samples from St James Burial Ground, London (1S17STJTT)

Report prepared by Hannah Ryan, University of Oxford, 2018 reviewed by Patrik Flammer and Adrian Smith

Methodology

From the preliminary assessment of the site, 31 soil samples were collected and analysed by microscopy. All soil samples were collected from the pelvic region of skeletons except for the one "control" sample of general cemetery soil. Skeletal samples came from 4 different trenches: 1 sample was from trench one, 8 from trench two, 17 from trench three and 4 from trench four. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 μl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed. Due to the low prevalence of eggs within single grave samples, 'eggs per gram' density was not calculated as the margin of error would be too high for these calculations to be meaningful.

Results and Interpretation

Eleven of thirty (36.67%) of the pelvic-soil samples contained at least one identifiable egg and no eggs were detected in the "control" sample. The most common parasite, found within 8 individuals, was *Ascaris* sp.. Other eggs identified included *Diphyllobothrium latum* (2 samples), a putative *Taenia* spp egg (1 sample), and a potential *Capillaria* specimen (1 sample) (see Figures 1-4 for examples, the scale bars represent 50 μm). To see which skeletal samples had identifiable eggs within the soil of their pelvic region, see Table 1. The nematode *Ascaris* sp. is transmitted via a faecal-oral route and is one of the most common found in archaeological samples. Today *Ascaris* sp. is prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, it is almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel). *Trichuris* sp. are regularly found to co-infect in regions where *Ascaris* sp. are prevalent so it is a surprise that no *Trichuris* sp. was found in these samples. The cestode *Taenia* spp. are associated with consumption of raw, undercooked or under cured red meat whilst *Diphyllobothrium* spp. are associated with the consumption of raw, undercooked or under cured fresh-water fish. One individual suffered from co-infection, Individual 4012 was infected with *Ascaris* sp. and the putative *Capillaria* spp.. Generally, eggs identified by microscopy are seen as a minimum estimate for the prevalence of helminths in the living population. The prevalence rates seen in the St James burial ground, London are comparable to many other archaeological sites, see figure 5 for a summary of the results.

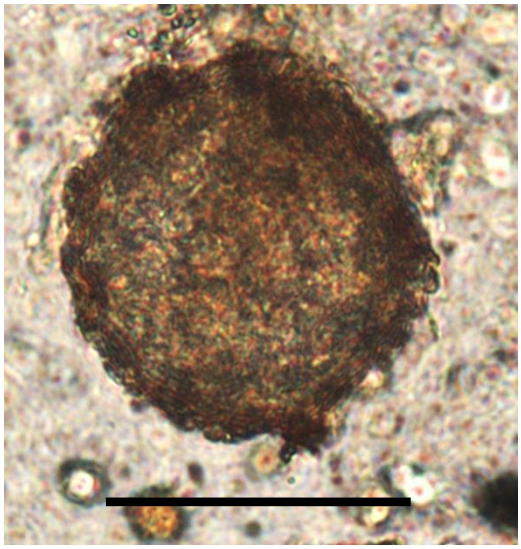


Figure 1: Ascaris sp. identified in sample 3021.

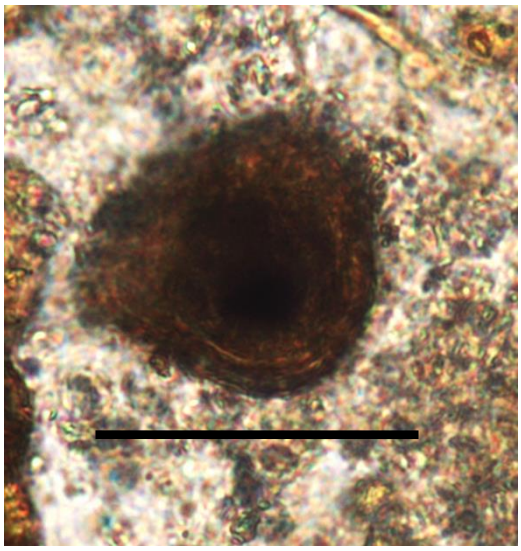


Figure 2: Putative Taenia spp. identified in sample 2119.

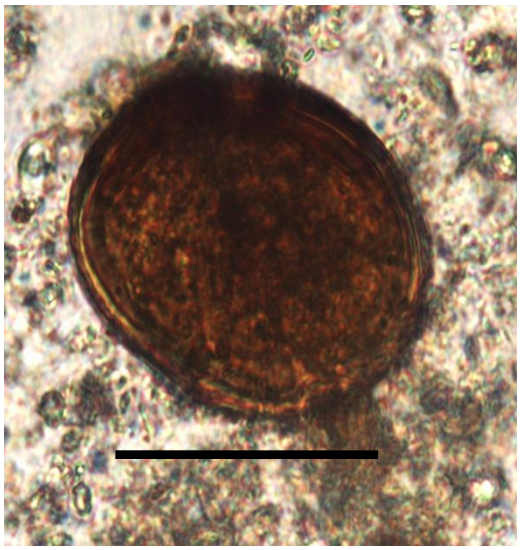


Figure 3: Diphyllobtohrium spp. identified in sample 2141.



Figure 4: Putative Capillaria specimen identified in sample 4012.

ID Number	Ascaris	Trichuris	Taenia	Diphyllobothrium	Eg (id in notes)	Total	Comments
1069						0	
3008						0	
3009						0	
3013						0	
3019						0	
3021	3					3	
3027	2					2	
3029						0	
3039						0	
3043						0	
3045	2					2	
3047						0	
3049						0	
3081						0	
3089						0	
3091						0	
3095	1					1	
3105						0	
2019						0	
2032	1					1	
2050						0	
2052	1					1	
2073						0	
2111				1		1	
2119			1			1	
2141				1		1	
4008						0	
4010	4					4	
4012	1				1	2	Potential capillaria
4016						0	
Control						0	

Table 1: The total number of eggs identified per sample after two rounds of microscopy.

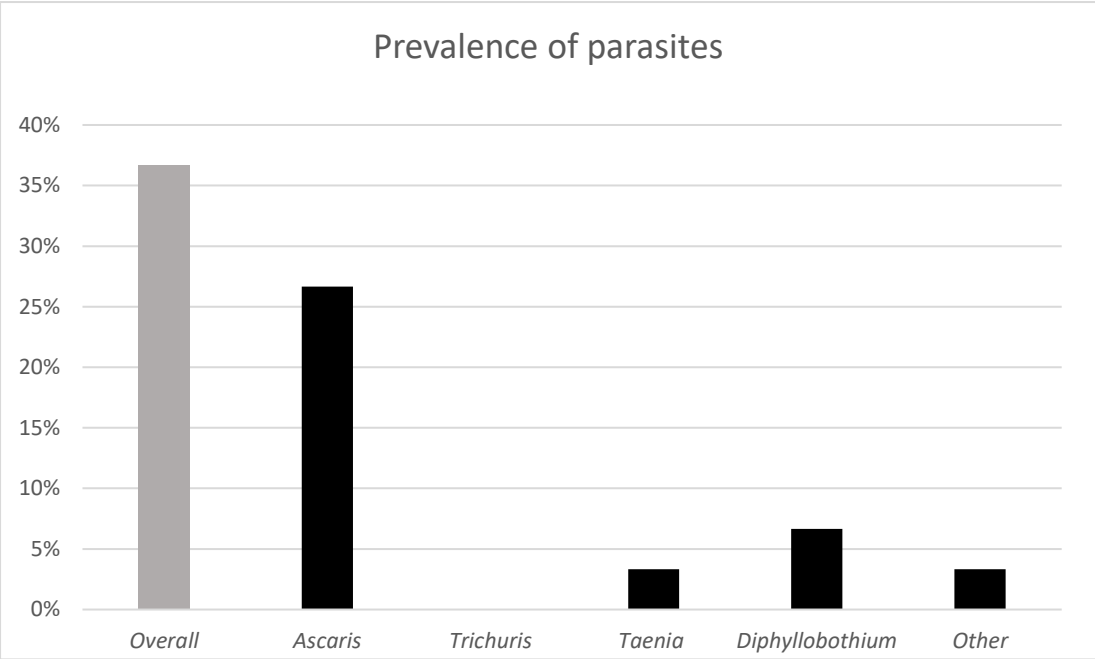


Figure 5: Overview of the prevalence of parasites.

Parasitological analysis of the samples from the Norman Chapter House, Worcester Cathedral

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

65 soil samples were collected and analysed by microscopy. All soil samples were collected from the pelvic region of skeletons and are believed to be Anglo Saxon in age. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results and Interpretation

Nine of sixty five (13.84%) of the soil samples contained at least one putative egg. The most common parasite, found within 6 individuals, was *Ascaris* sp.. Other eggs identified included *Trichuris* sp. (1 sample), *Diphyllobothrium latum* (1 sample), a potential *Enterobius* sp. (1 sample) and, a putative unidentified egg (see Figures 1-3 for microscopy examples). A full description of which skeletal samples had identifiable eggs within the soil of their pelvic region is provided (Table 1). The nematodes *Ascaris* and *Trichuris* sp. are transmitted via a faecal-oral route and represent the most common helminths found in archaeological samples. Today both *Ascaris* and *Trichuris* sp. are prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, they are almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel). Infection with *Enterobius vermicularis* is a quite common infection throughout the world (particularly targeting children or their parents). Meanwhile the cestode *Diphyllobothrium latum* is associated with the consumption of raw, undercooked or under cured fresh-water fish. One individual suffered from co-infection. Sample 130 had both *Ascaris* sp. and a structure that represents a putative helminth (worm) egg (but not identifiable to a particular genus). Generally, eggs identified by microscopy are seen as a minimum estimate for the prevalence of helminths in the living population. The prevalence rates seen in the Norman Chapter House, Worcester Cathedral, is low in comparison to other archaeological sites however, the values are still high enough that a population of helminths could be maintained. See Figure 4 for a summary of the results.

Prevalence rates were also assessed against skeletal traits provided. In modern populations, children are more likely to be infected with parasites transmitted by the fecal-oral route than adults however in the samples from Worcester Cathedral, there did not seem to be a significant difference between age group and prevalence rate (see Figure 5). Individuals just identified as adult was placed in the 18-35 age category. In addition, there are multiple modern case examples of differential risk based on sex. These are usually based on behavioural differences. In the samples from Worcester there seems to be a slightly higher risk of infection for males in comparison to females (see Figure 6). Considering the overall low prevalence rates, it is difficult to say whether the patterns detected reflect sampling bias or the living infection loads.

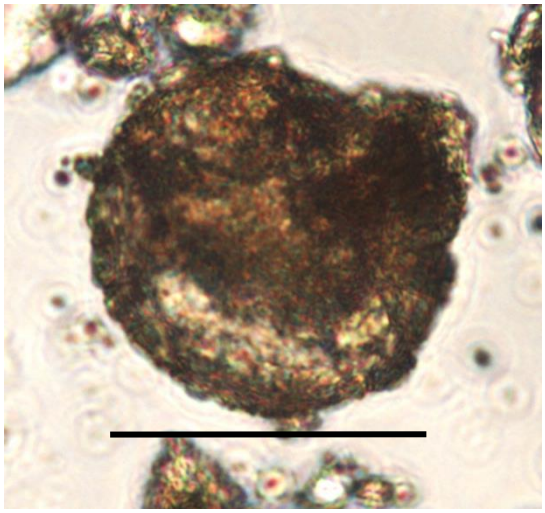


Figure 1: *Ascaris* sp. identified in sample 82 (scale bars represent 50 μm).

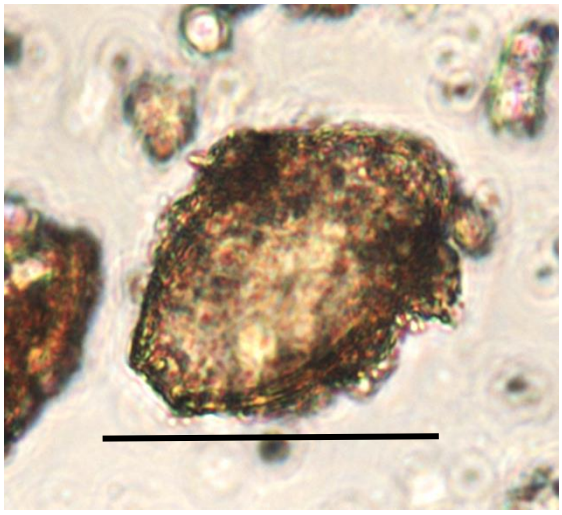


Figure 2: *Trichuris* sp. identified in sample 197 (scale bars represent 50 μm).

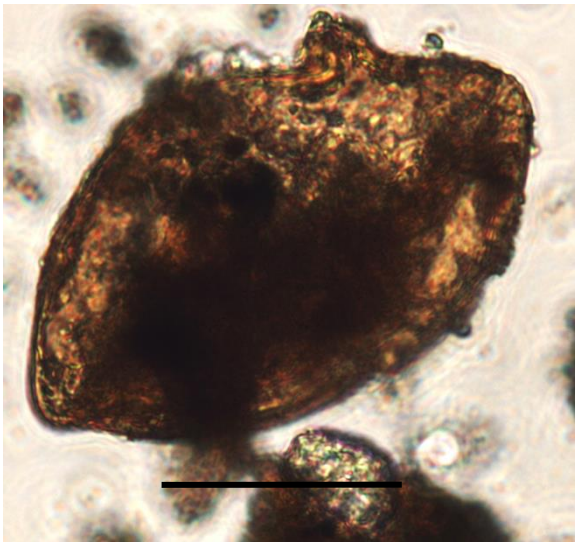


Figure 3: *Putative worm egg* identified in sample 20255 (scale bars represent 50 μm).

ID Number	Ascaris	Trichuris	Taenia	Diphyllobothrium	Egg (id in notes)	Total	Comments
37						0	
41						0	
42						0	
44						0	
49						0	
50						0	
52	1					1	
58						0	
62						0	
64						0	
66						0	
68						0	
71						0	
72						0	
73						0	
82	1					1	
85				1		1	
86						0	
89						0	
90						0	
95						0	
99						0	
100						0	
101						0	
102						0	
106						0	
107	1					1	
113						0	
114						0	
118						0	
119						0	
122						0	
123						0	
125						0	
130	1				1	2	Putative helmith egg
131						0	
132						0	
133						0	
135						0	

137						0	
142						0	
143						0	
149						0	
152						0	
157						0	
165	1					1	
166						0	
167						0	
170					1	1	Potential *Enterobius*
172						0	
173						0	
180						0	
181						0	
182						0	
183						0	
184						0	
185						0	
187						0	
193						0	
194						0	
195						0	
196						0	
197		1				1	
199	1					1	

Table 1: The total number of eggs identified per sample after two rounds of microscopy.

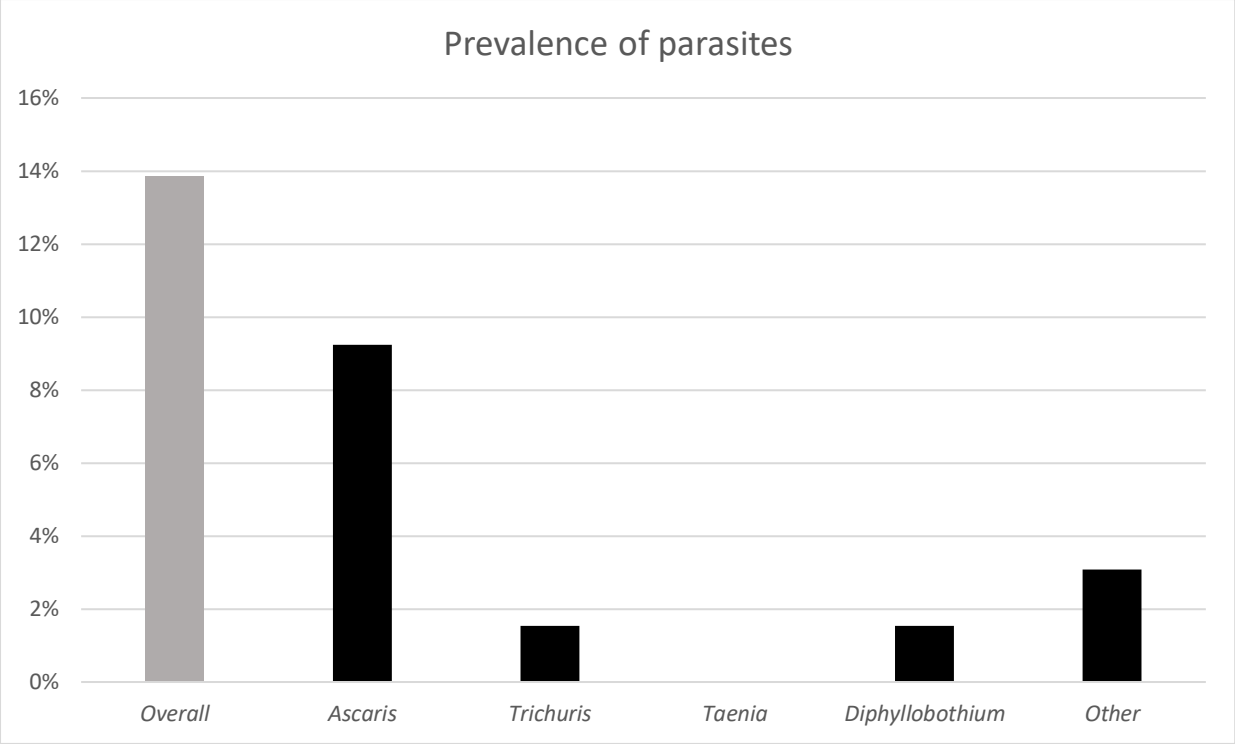


Figure 4: Overview of the prevalence of parasites.

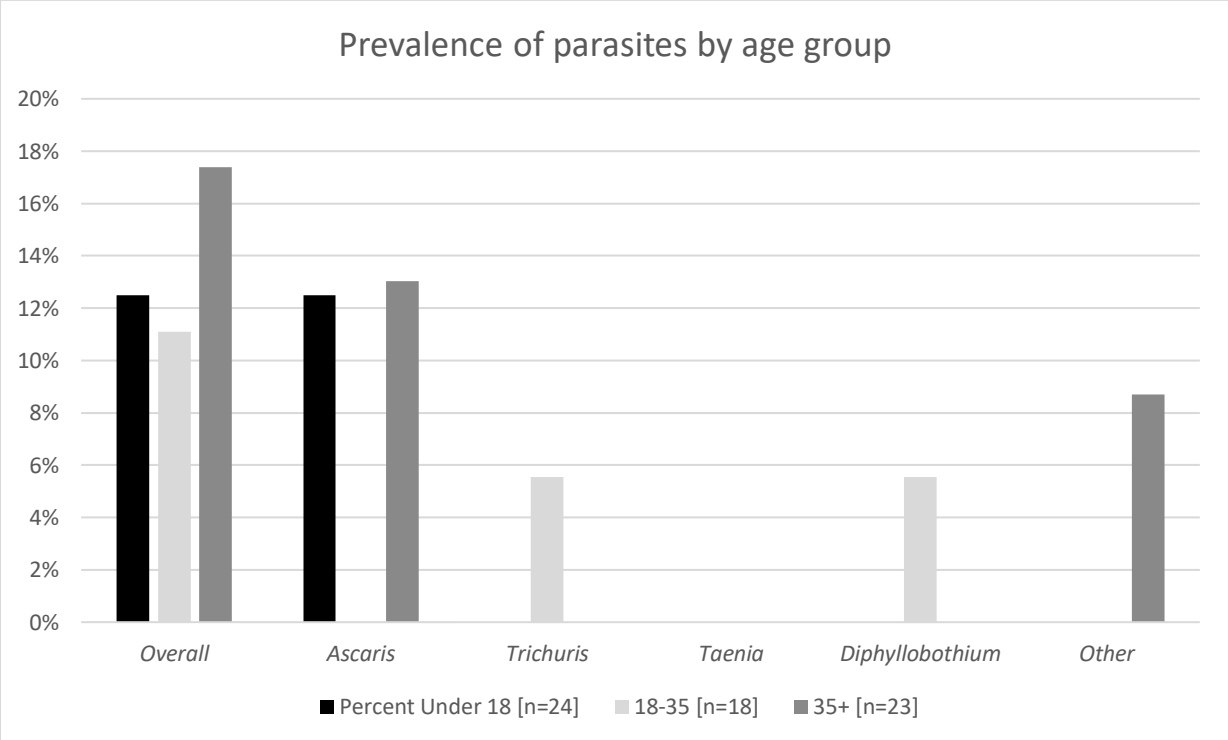


Figure 5: Overview of the prevalence of parasites by age group.

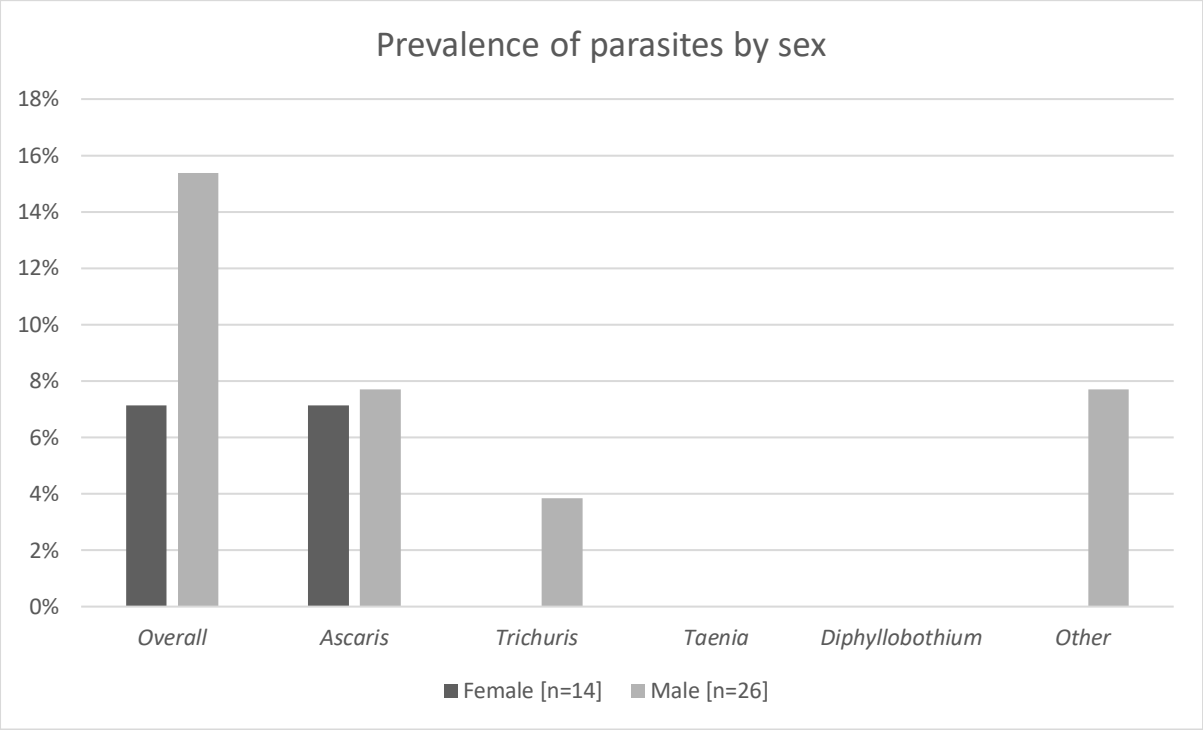


Figure 6: Overview of the prevalence of parasites by sex.

Parasitological analysis of the samples from Christchurch Priory visitors Centre, Dorset (119070).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

Ten samples were collected for microscopy however only eight were analysed due to some of the vessels getting damaged in the post (samples 218 and 334). All samples were collected from the pelvic region of Medieval skeletons. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample (using 20 µl sub-sample per round, 40 µl in total) using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

After 2 rounds of microscopy, no helminth eggs were identified. We would expect the Medieval population to suffer from helminth infections however, based on modern prevalence rates (often <20%) there is a chance that helminths would go undetected in ten samples.

Parasitological analysis of the samples from Riding Court Farm, Datchet (106002).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

One sample was collected from the pelvic region of a Prehistoric skeleton and analysed by microscopy. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample (using 20 µl sub-sample per round, 40 µl in total) using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

After 2 rounds of microscopy, no helminth eggs were identified. We would expect the Prehistoric population to suffer from helminth infections however, based on modern prevalence rates (often <20%) there is a high chance that helminths would go undetected in a single sample.

Parasitological analysis of the samples from Wentworth Drive, land adjacent, Bleadon (115540).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

Nine samples were collected and analysed by microscopy. All samples were collected from the pelvic region of Roman skeletons. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample (using 20 µl sub-sample per round, 40 µl in total) using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

Six out of nine (66.67%) of the pelvic-soil samples contained at least one putative helminth egg. The most common helminth (parasitic worm), found within six individuals, was *Ascaris* sp.. Other eggs identified included a putative Trematode spp. (1 sample) and, a putative unidentified helminth egg (1 sample) (see Figures 1 and 2 for examples). To see which skeletal samples had identifiable eggs within the soil of their pelvic region, see Table 1. The nematode (Roundworm) *Ascaris* sp. is transmitted via a faecal-oral route and is one of the most common found in archaeological samples. Today *Ascaris* sp. is prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, it is almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel). *Trichuris* sp. are regularly found to co-infect in regions where *Ascaris* sp. are prevalent so it is a surprise that no *Trichuris* sp. was found in these samples. Generally, eggs identified by microscopy are seen as a minimum estimate for the prevalence of helminths in the living population. The prevalence rates seen in Wentworth Drive, Bleadon, are high but this may be due to the size of the site, see Figure 3 for a summary of the results.

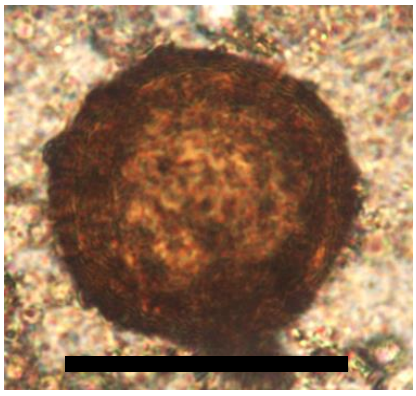


Figure 1: *Ascaris* sp. identified in sample 1084 (scale bars represent 50 µm).

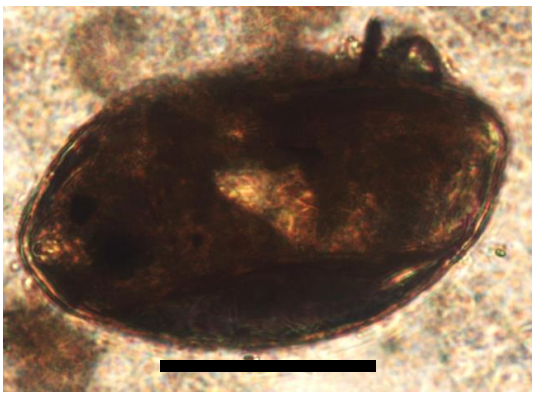


Figure 2: Putative Trematode spp. identified in sample 1084 (scale bars represent 50 µm).

ID number	*Ascaris*	*Trichuris*	*Taenia*	*Diphyllobothrium*	Unidentified Egg	Total	Comments
1042						0	
1045						0	
1039	2				1	3	Unidentified
1073	3					3	
1067	1					1	
1079	2					2	
1084	1				1	2	Putative Trematode spp.
1443	3					3	
1535						0	

Table 1: The total number of eggs identified per sample after two rounds of microscopy.

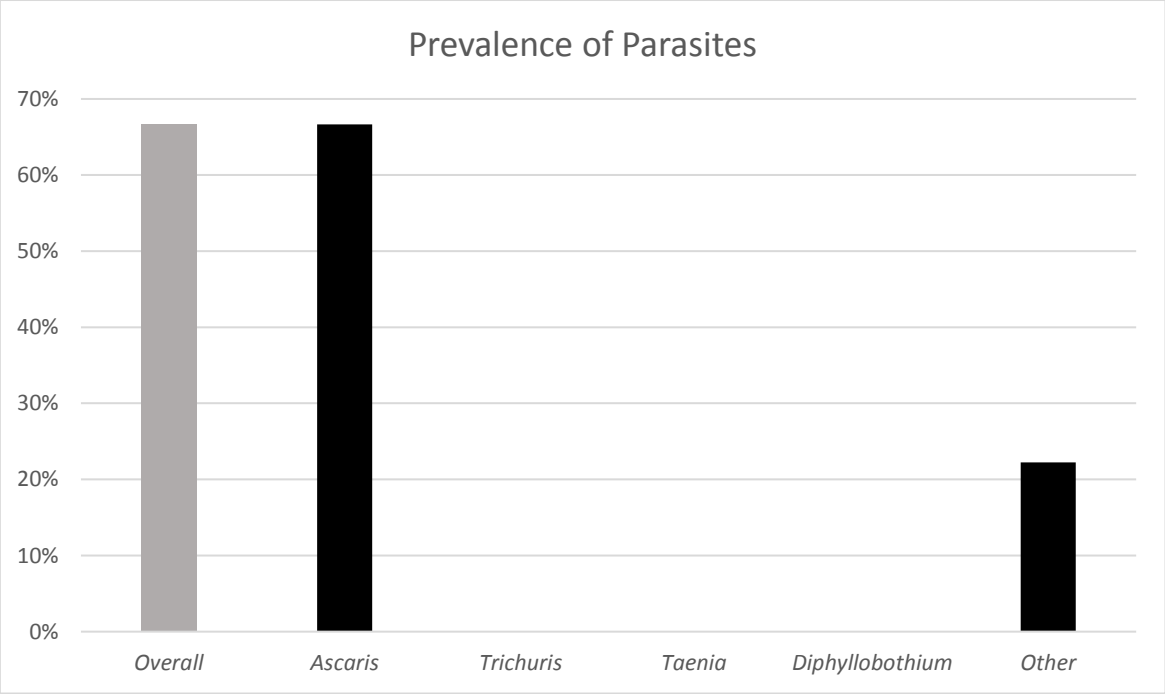


Figure 3: Overview of the prevalence of parasites.

Parasitological analysis of the samples from Bulford Camp to Tidworth cable watching, Salisbury (111422).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

Two samples were collected and analysed by microscopy. All samples were collected from the pelvic region of Prehistoric skeletons. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample (using 20 µl sub-sample per round, 40 µl in total) using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

Sample 16 had one identified egg, a putative decorticated *Ascaris* sp.. The egg was partially obscured so there is lower confidence in this identification (see Figure 1). The nematode *Ascaris* sp. is transmitted via a faecal-oral route and is one of the most common helminths found in archaeological samples. Today *Ascaris* sp. are prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, they are almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel).

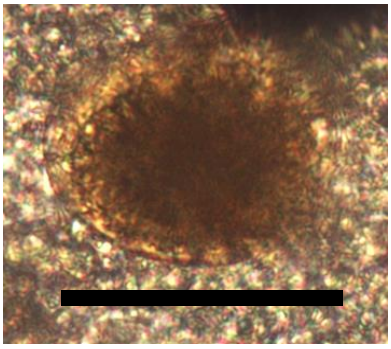


Figure 1: Putative *Ascaris* sp. identified in sample 1084 (scale bars represent 50 µm).

Parasitological analysis of the samples from Chapel Riverside, Southampton (107173).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

Sixty-nine samples were collected for microscopy however only fifty-two were analysed due to some of the vessels getting damaged in the post. Samples 2138, 2184, 2190, 2203, 2238, 2266, 2287, 2444, 2553, 2565, 2577, 2592, 2595, 2612, 2618, 2624 and 2683 were excluded to prevent the issue of cross contamination. All samples were collected from the pelvic region of Medieval skeletons. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample (using 20 µl sub-sample per round, 40 µl in total) using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

21 out of 52 (40.38%) of the pelvic-soil samples contained at least one putative helminth egg. The most common helminth (parasitic worm), found within 20 individuals, was *Ascaris* sp.. Other eggs identified included *Taenia* sp. eggs (1 sample), a putative *Schistosoma* spp. (1 sample), and putative unidentified helminth eggs (2 samples) (see Figures 1-3 for examples). To see which skeletal samples had identifiable eggs within the soil of their pelvic region, see Table 1. The nematode (Roundworm) *Ascaris* sp. is transmitted via a faecal-oral route and is one of the most common helminths found in archaeological samples. Today both *Ascaris* sp. is prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, they are almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel). *Trichuris* sp. are regularly found to co-infect in regions where *Ascaris* sp. are prevalent so it is a surprise that no *Trichuris* sp. was found in these samples. The cestode (Tapeworm) *Taenia* spp. are associated with consumption of raw, undercooked or under cured red meat. Generally, eggs identified by microscopy are seen as a minimum estimate for the prevalence of helminths in the living population. The prevalence rates seen in Chapel Riverside, Southampton, are comparable to other archaeological sites, see Figure 4 for a summary of the results.



Figure 1: *Ascaris* sp. identified in sample 2120 (scale bars represent 50 µm).

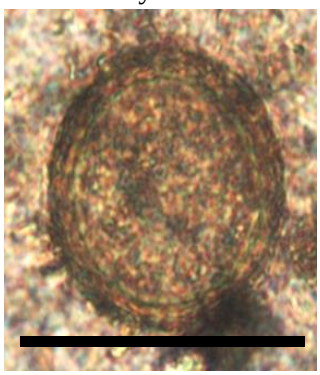


Figure 2: Putative *Taenia* spp. identified in sample 2617 (scale bars represent 50 µm).



Figure 3: Putative *Schistosoma* spp. identified in sample 2010 (scale bars represent 50 µm).

ID number	*Ascaris*	*Trichuris*	*Taenia*	*Diphyllobothrium*	Unidentified Egg	Total	Comments
2010	1				1	2	*Schistosoma* spp.
2019	1					1	
2033						0	
2037						0	
2044	1					1	
2056	1				1	2	Unidentified egg
2059	3					3	
2086	1					1	
2088						0	
2101	1					1	
2103						0	
2120	1					1	
2130	1					1	
2135						0	
2153	1					1	
2159						0	
2173						0	
2181						0	
2185						0	
2192	3					3	
2207	1					1	
2213	1					1	
2225						0	
2240						0	
2266						0	
2271	1					1	
2289						0	
2310	2					2	
2314	1					1	
2329	1					1	
2342						0	
2351						0	
2357						0	
2373						0	
2379	1					1	
2400						0	
2409						0	
2417						0	
2425						0	

2432						0	
2438						0	
2446						0	
2462						0	
2468						0	
2479						0	
2518	1					1	
2562						0	
2567						0	
2593					1	1	Unidentified egg
2604						0	
2617	1		1			2	
2639						0	

Table 1: The total number of eggs identified per sample after two rounds of microscopy.

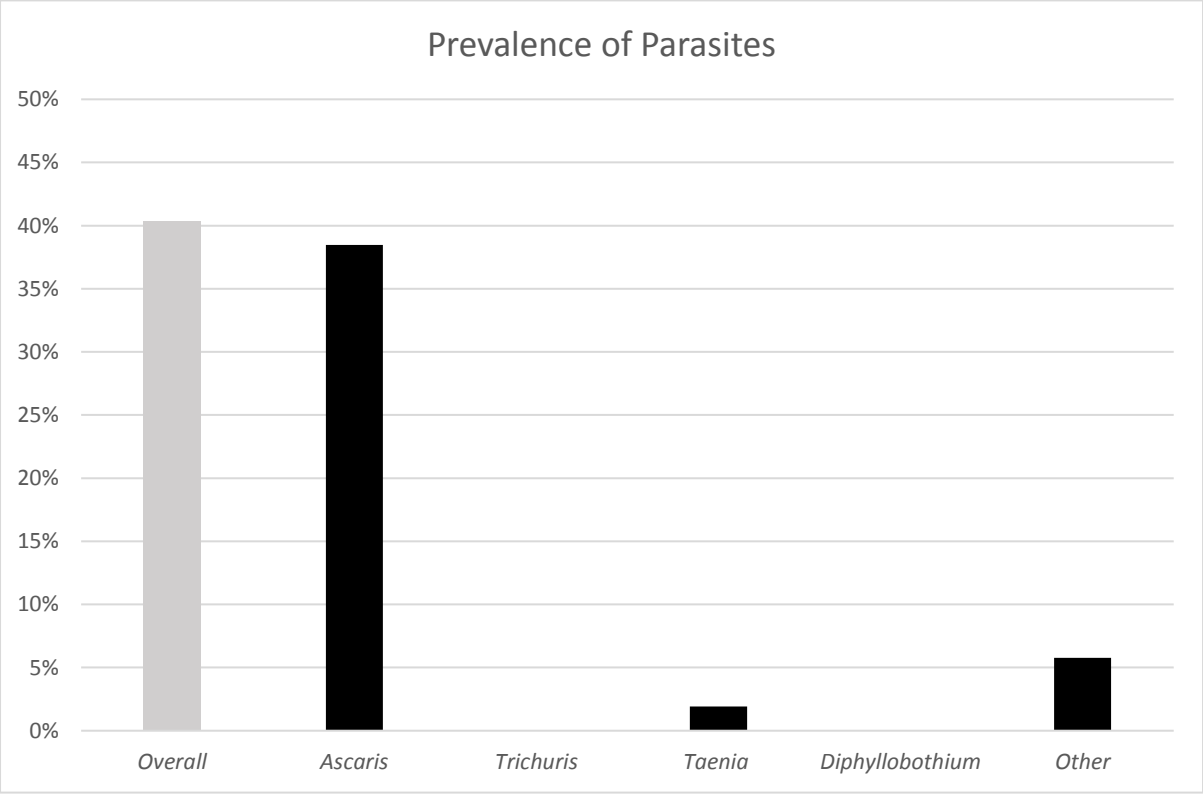


Figure 4: Overview of the prevalence of parasites.

Parasitological analysis of the samples from Long Lane, Southwark, London (51591).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

Two soil samples were collected from Medieval ditches suspected to be used for refuse and analysed by microscopy. The samples were 529 (from context 1711) and 531 (context 1719). To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

Whilst sample 531 contained no identifiable helminth eggs, sample 529 had identifiable *Trichuris* sp. eggs (288.46 eggs per gram). The nematode *Trichuris* sp. is transmitted via a faecal-oral route and is one of the most common helminths found in archaeological samples. Today *Trichuris* sp. are prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, they are almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel). *Ascaris* sp. are regularly found to co-infect in regions where *Trichuris* sp. are prevalent therefore it is unusual that no *Ascaris* sp. was found in these samples.



Figure 1: *Trichuris* sp. identified in sample 529 (scale bars represent 50 µm).

Parasitological analysis of the samples from St Mary's Axe, London (AMX07/64780).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

Six soil samples were collected from Medieval back filled pits and analysed by microscopy. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

Four of six samples contained at least one putative egg. The only identified parasite was *Trichuris* sp.. The numbers of *Trichuris* sp. eggs are given in Table 1. The nematode *Trichuris* sp. is transmitted via a faecal-oral route and is one of the most common helminths found in archaeological samples. Today *Trichuris* sp. are prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, they are almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel). *Ascaris* sp. are regularly found to co-infect in regions where *Trichuris* sp. are prevalent but no *Ascaris* sp. eggs were found in these samples.

Sample ID	*Trichuris* sp. [per g]
AMX1	0
AMX2	0
AMX6	188.68
AMX7	300
AMX8	178.57
AMX21	94.34

Table 1: Numbers of *Trichuris* sp. eggs detected in each sample.

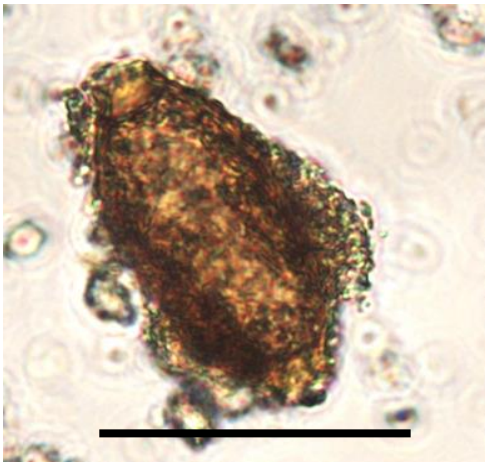


Figure 1: *Trichuris* sp. identified in sample AMX7 (scale bars represent 50 µm).

Parasitological analysis of the samples from SSSM, Churchill (110766).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

One soil sample (number 116) was collected from the pelvic region of a Roman skeleton and was analysed by microscopy. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

Sample 116 had one identified egg, a putative *Ascaris* sp. with a poor structural condition. The nematode *Ascaris* sp. is transmitted via a faecal-oral route and is one of the most common helminths found in archaeological samples. Today *Ascaris* sp. are prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, they are almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel).

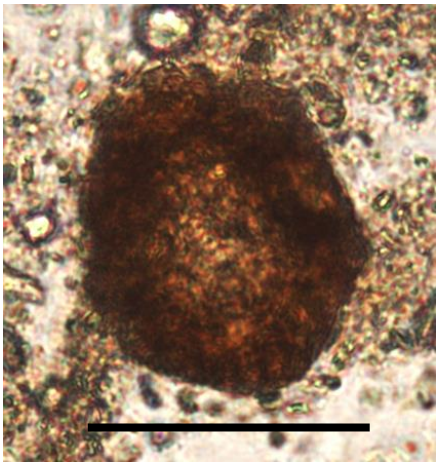


Figure 1: Putative *Ascaris* sp. identified in sample 116 (scale bars represent 50 µm).

Parasitological analysis of the samples from Larkhill, Durrington (113931, 109516, 113936).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

7 samples were collected and analysed by microscopy. All samples were collected from the pelvic region of skeletons but, from three different phases of excavation. 2 prehistoric samples were collected from excavation 113931, 4 samples, suspected to date between the Late Neolithic and Early Bronze Age, from excavation 109516 and, 1 sample from 113936, which is of an undetermined age anywhere between the Prehistoric and Medieval. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

One of the seven (14.29%) pelvic soil samples contained at least one putative egg. None were recovered from excavations 113931 or 109516 but 1 was recovered from the only sample from excavation 113936. The most commonly identified helminths in the archaeological record (*Ascaris* sp. and *Trichuris* sp.) were not identified in any of the samples. Sample 521 from excavation 113936 had a putative hookworm egg, potentially from the genus *Ancylostoma*.

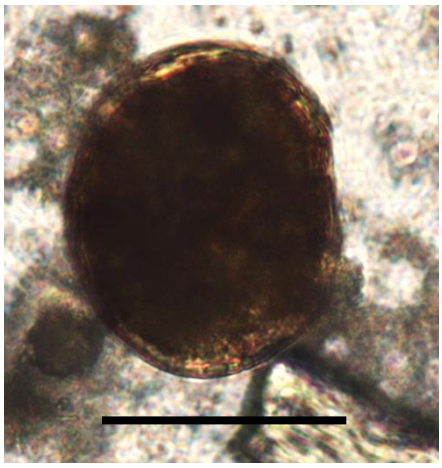


Figure 1: Putative hookworm egg identified in sample 521 from excavation 113936 (scale bars represent 50 µm).

Parasitological analysis of the samples Bulford, Salisbury (113930).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

One soil sample was collected from the pelvic region of a Bronze Age skeleton and analysed by microscopy. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

Sample 1170 from Bulford had three identifiable eggs. Two have been identified as a possible *Taenia* sp. and one is a putative egg of unknown origin, see Figures 1-2. The cestode *Taenia* sp. are associated with the consumption of raw, undercooked or under cured red meat.

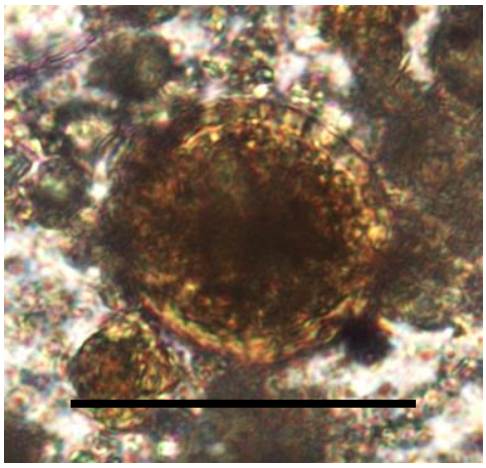


Figure 1: Putative *Taenia* sp. identified in sample 1170 (scale bars represent 50 µm).

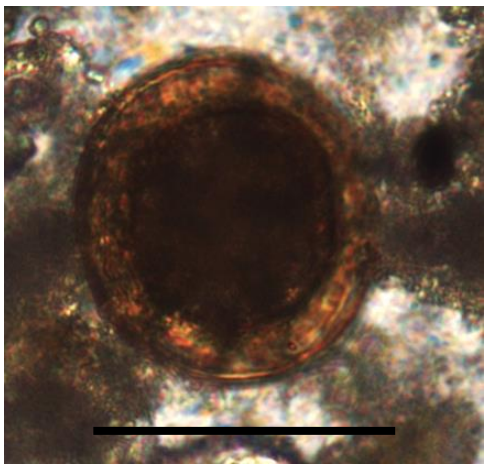


Figure 2: Putative helminth egg identified in sample 1170 (scale bars represent 50 µm).

Parasitological analysis of the samples Datchet, Slough (106001).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

Three samples were collected and analysed by microscopy. All samples were collected from the pelvic region of Bronze Age skeletons. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

After 2 rounds of microscopy, no helminth eggs were identified in these samples. Bronze Age populations are likely to suffer from helminth infections however, based on modern prevalence rates (often <20%) there is a high chance that helminths would go undetected in 3 samples.

Parasitological analysis of the samples Razor's Farm, Basingstoke (74586).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

One sample was collected from a Roman cess pit and analysed by microscopy. To prepare the sample 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

No eggs were detected in this sample. This is very unusual for samples from cess pit or other communal deposits which often contain multiple detectable parasite eggs. One sample may not give results representative for this location.

Parasitological analysis of the samples Vale Crematorium, Worcestershire (114416).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

One sample was collected from the pelvic region of a Bronze Age skeleton and analysed by microscopy. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

After 2 rounds of microscopy, no helminth eggs were identified. We would expect the Bronze Age population to suffer from helminth infections however, based on modern prevalence rates (often <20%) there is a high chance that helminths would go undetected in a single sample.

Parasitological analysis of the samples Taplow Quarry, Maidenhead (61058).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

One sample was collected from the pelvic region of a Prehistoric skeleton and analysed by microscopy. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

After 2 rounds of microscopy, no helminth eggs were identified. We would expect the Prehistoric population to suffer from helminth infections however, based on modern prevalence rates (often <20%) there is a high chance that helminths would go undetected in a single sample.

Parasitological analysis of the samples North Park Quarry, Bletchingley (86772).

Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Three samples were collected and analysed by microscopy. All samples were collected from the pelvic region of Roman skeletons. To prepare the sample for microscopy, 5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 µl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results

After 2 rounds of microscopy, no helminth eggs were identified in these samples. We would expect the Roman population to suffer from helminth infections however, based on modern prevalence rates (often 30-40%) and any disturbance that can be found in the archaeological record, there is a high chance that helminths would go undetected in 3 samples.

Parasitological analysis of the samples from All Saints in the Marsh, Peasholme; the former Haymarket, York

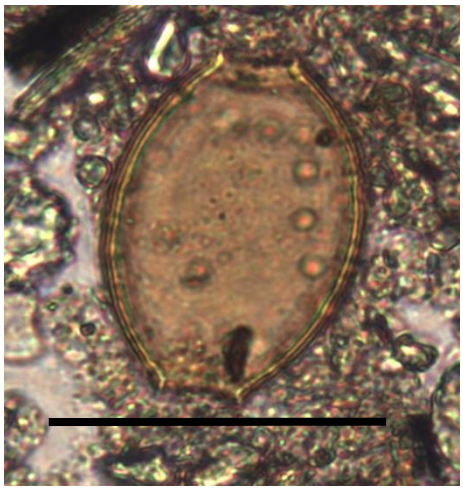
Report prepared by Hannah Ryan, University of Oxford, 2018, reviewed by Patrik Flammer and Adrian Smith.

Methodology

36 soil samples were collected and analysed by microscopy. All soil samples were collected during the post excavation cleaning phase, with soil predominantly collected from the pelvic region of the skeletons. All samples are believed to be Medieval in age except sample 84536 (SK382), which is believed to date to the Roman period. To prepare the sample for microscopy, 4.5-7 g of soil was weighed and mixed with 20 ml of MilliQ-water. The sample was vortexed and left over night to disaggregate. A subsample of 500 μl was then taken for microscopy. Two rounds of microscopy were carried out per sample using light microscopy on a Nikon Eclipse E400 with Nikon 10x/0.25 Ph1 DL and 40x/0.65 Ph2 DL objectives. No further processing was carried out on the samples however they were agitated before pipetting to stop denser material settling. A QImaging MP5.0 RTV camera was used with the software QImaging QCapture pro to record any suspected eggs in a sample and these images were assessed against reference images before the final count number was confirmed.

Results and Interpretation

Seventeen of thirty six (47.22%) of the soil samples contained at least one putative egg. The most common parasite, found within 14 individuals, was *Ascaris* sp.. Other eggs identified included *Trichuris* sp. (3 samples), and a putative unidentified egg (see Figures 1-2 for microscopy examples). A full description of which skeletal samples had identifiable eggs within the soil of their pelvic region is provided (Table 1). The nematodes *Ascaris* sp. and *Trichuris* sp. are transmitted via a faecal-oral route and they are the most common helminths found in archaeological samples. Today both *Ascaris sp.* and *Trichuris* sp. are prevalent in many parts of the developing world particularly those with issues around sanitation infrastructure however, they are almost completely absent from modern Europe (all diagnosed infections in the UK are considered as associated with foreign travel). One individual suffered from co-infection, sample 84357 had both *Ascaris* and *Trichuris* sp. present. Prevalence rates were also assessed against skeletal traits provided. Age of skeleton seemed to be a predictor of infection with juvenile individuals being more likely to host a parasite, predominantly *Ascaris* and *Trichuris* sp. (see Figure 3). In modern populations, children are also more likely to be infected with parasites transmitted by the fecal-oral route. Generally, eggs identified by microscopy are seen as a minimum estimate for the prevalence of helminths in the living population. The prevalence rates seen in All Saints Peasholme, York are comparable to many other archaeological sites, see Figure 4 for a summary of the results.



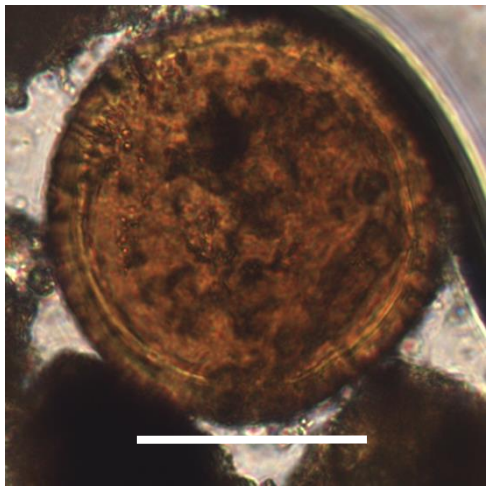


Figure 1: *Trichuris* sp. identified in sample 84357 (scale bars represent 50 μm).

Figure 2: An unidentified egg in sample 83834 (scale bars represent 50 μm).

ID Number	Ascaris	Trichuris	Taenia	Diphyllobothrium	Unidentified Egg	Total
83512	2					2
83578	1					1
83582		1				1
83676						0
83678	2					2
83704						0
83710	1					1
83766						0
83806						0
83834					1	1
83873						0
83876						0
83914	4					4
83987						0
84023	1					1
84032	2					2
84108						0
84168						0
84201						0
84255						0
84330	1					1
84342	2					2
84357	1	1				2
84387						0
84396						0
84419						0
84422		2				2
84425						0
84428	2					2
84431	2					2
84533						0
84536	1					1
84557	1					1
84560						0
84638						0
84692						0

Table 1: The total number of eggs identified per sample after two rounds of microscopy.

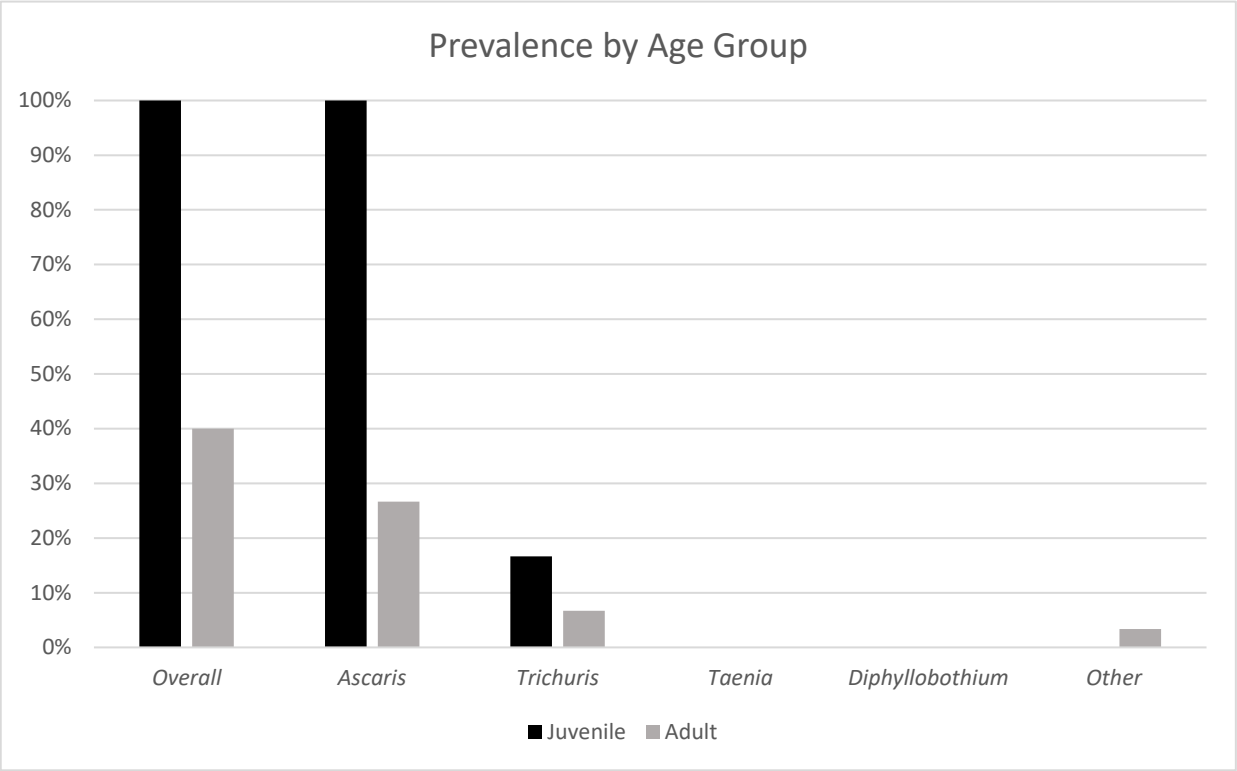


Figure 3: Overview of the prevalence of parasites by age group.

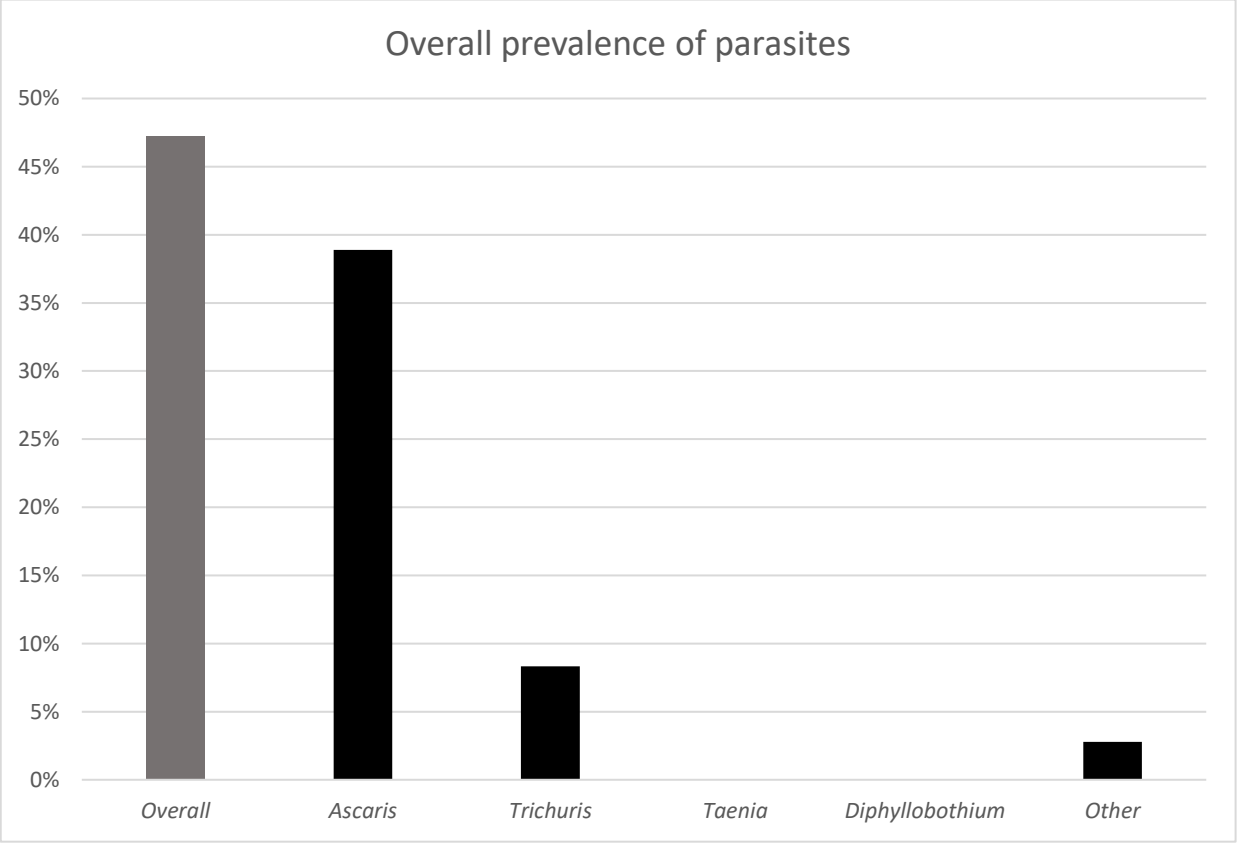


Figure 4: Overview of the prevalence of parasites.

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Predicting the viability of archaic human hybrids using a mitochondrial proxy

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Predicting the viability of archaic human hybrids using a mitochondrial proxy

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Abstract

Ancient DNA evidence has demonstrated a complex pattern of admixture between humans and several hominin lineages. Many segments of the modern human genome are devoid of Neanderthal ancestry, however, and this non-random distribution has raised questions regarding the frequency and fitness effects of hominin admixture events. Here, we test the hypothesis that hominin hybrid offspring suffered a reduction in fertility by developing a genetic sequence divergence proxy (using both mitochondrial and nuclear DNA) using numerous pairs of terrestrial mammals capable of producing hybrid offspring. Our results reveal a threshold separating species pairs whose divergence values fall within two categories: those whose hybrid offspring can successfully reproduce without backcrossing with their parent species, and those whose hybrid offspring cannot. From this we infer that the potential offspring between Neanderthals, Denisovans, the ancient individuals from the Sima de los Huesos and anatomically modern humans would not have suffered a reduction in fertility. The genetic distance proxy developed in this study can also be employed to predict the hybridibilty of any two mammalian species.

Introduction

Though numerous interspecies hybrids have been recorded, predicting their viability and relative fertility has proved difficult. For example, the absence of Neanderthal mitochondrial genomes in the modern human population led some to suggest that anatomically modern humans (AMH) and Neanderthals did not hybridise [1–3]. Analyses of whole genome sequences derived from ancient individuals, however, have demonstrated that archaic hominins including Neanderthals and Denisovans did in fact hybridise not only with AMH [4–6] but also with each other [7]. For many anthropologists, these results corroborated previous conclusions (based on

morphological grounds) that Neanderthals and AMH could have produced fertile offspring (*e.g. [8]*).

A recent study [9], however, conflated the observation of genetic deserts devoid of Neanderthal ancestry on modern human autosomes, and especially the X chromosome, with reproductive incompatibilities between humans and Neanderthal. The authors also suggested that demographic factors likely played a role in the formation of these deserts, and three additional studies [10–12] concluded that most selection against Neanderthal DNA in genic regions was not due to reproductive incompatibilities, but was instead the result of the Neanderthals' high genetic load. In addition, the initially high proportion of Neanderthal admixture [13] suggests that early male and female hybrids of AMH and Neanderthals were normally fertile, and that any Neanderthal variants that may have interfered with fertility within an AMH genetic background were likely confined to a few genetic loci. Lastly, multiple factors including ecological drivers can lead to genomic deficits of introgression within hybrid offspring that are otherwise fertile [14], thus suggesting that the existence of genetic deserts alone does not necessarily imply reproductive incompatibility.

More generally, assessing whether any two species are capable of producing viable or fertile offspring is difficult in the absence captive breeding experiments or field data. In addition, there has been no robust, quantitative biological measure of the expected breeding capability of hybrid offspring, and thus no rigorous method to ascertain the specific viability of archaic human hybrids, or the potential for hybridisation between more ancient hominin lineages. Determining whether distinct hominin lineages were capable of producing fertile offspring is important not only for understanding the patterns of hybrid ancestry within modern humans, but also for ascertaining the degree to which reproductive isolation (as a result of either cultural or biological phenomena) existed between AMH and other hominin lineages.

Though numerous studies have speculated about the potential for hominin lineages to produce viable hybrids, few have made use of the numerous pairs of mammalian species known to produce hybrid offspring along a continuum of male (and to a

lesser extent, female) fertility. Based on the qualitative correlation that the fertility of hybrids generally decreases with divergence time, two recent studies [15,16] speculated that, given their relatively recent temporal divergence, AMH and Neanderthals could have retained the ability to produce fertile offspring. These studies could not rule out the fact that the male hybrids may have possessed lower fertility as a result of Haldane's Rule which states that if one sex is rare, absent or sterile among hybrid offspring, it is the heterogametic sex.

In order to obtain a quantitative measure of whether two lineages can interbreed, we developed a framework based on the correlation between mitochondrial genetic distance between mammalian species pairs, and the manifestation of hybrid sterility. Some have questioned this approach and stated that measures of species divergence are not necessarily reliable predictors of hybrid sterility [17,18]. Other synthetic studies have reported that genetic divergence values do in fact generally correlate with species boundaries [19], and a specific study of damselflies demonstrated a strong correlation between the genetic distances between species pairs and their relative reproductive isolation [20].

For this study, we first explicitly defined two dichotomous categories along the spectrum of hybrid compatibility. Category 1 is defined by terrestrial mammalian species pairs capable of producing F1 offspring that can reproduce without backcrossing with a parent species (even if there are observed asymmetries in gene flow and variation in male fertility amongst the hybrids) (Table S1). Category 2 is defined by pairs of species that can produce viable F1 offspring, but these hybrids either cannot reproduce without backcrossing with a parent species or are completely infertile (Table S1). We determined the categorical assignment of each species pair (Table S1, Fig. S1) by following a flowchart (Fig. S2) based upon empirical evidence derived from experimental studies of F1 hybrid fertility. We were able to place eight species pairs into Category 1, eight into Category 2, and we further compiled 17 species pairs known to produce viable offspring, but for which there was insufficient evidence to confidently assign them into either category (Table S2, Fig. S3).

We then obtained published sequences across all species (Table S3) of both the cytochrome b gene (*CYTB*) (n=1795) and mitochondrial genomes (n=30) (excluding the control region) from multiple individuals per species. We ensured that the selected mitochondrial sequences for each species were neither mislabelled, nor nuclear copies of mitochondrial genes (NUMTs), nor derived from hybrid populations by matching the phylogenies to nuclear species trees. We then calculated average pairwise genetic distances between each species from the sequence alignments using both Hamming (raw) distances and evolutionary models. In addition, we calculated genetic distances for four nuclear loci (*CHRNA1*, *GHR*, *ZFX*, *ZFY*) between ten primate species pairs [21] (Fig. S4)

Results and Discussion

The calculated divergence estimates using *CYTB* revealed a clear threshold, with a small overlap, between the two categories (Fig 1, Fig. S1). More specifically, none of the eight Category 1 pairs that were capable of producing fully fertile offspring possessed *CYTB* sequences with raw average pairwise divergence values greater than 7.86%. Other than a pair of vole species that possessed a divergence value of 7.54%, all other Category 2 pairs possessed divergence values equal or greater than 8.02%. The existence of a genetic distance threshold separating the two categories also held true for the mitogenomes. In addition, the hybrid offspring of the two most divergent pairs—Rhesus Macaque (*Macaca mulatta*) x Hamadryas Baboon (*Papio hamadryas*) and Domestic Pig (*Sus domesticus*) x Babirusa (*Babyrousa celebensis*)—were shown to be completely infertile, corroborating previous studies showing that along the continuum of speciation, infertility evolves prior to inviability [22–24]. In addition, a Mann-Whitney U test showed significantly lower genetic divergence values of species pairs in Category 1 relative to those in Category 2 ($p < 6.3e-4$).

Importantly, the two categories of fertility defined here are not strictly linked with gene flow. For instance, though both male and female hybrid offspring of all species

pairs in Category 1 can reproduce without requiring a backcross with a parent species, gene flow asymmetries have been demonstrated in virtually all Category 1 pairs including two species of house mice (*Mus musculus musculus* x *Mus musculus domesticus*) [25], and between brown bears (*Ursus arctos*) and polar bears (*Ursus maritimus*) [14] (Table S1). Gene flow has also been demonstrated between Category 2 species (including *Mus musculus* and *M. spretus [26]*) whose F1 hybrid offspring cannot produce viable F2s. Since both fertility and the potential for gene flow vary along a continuum, it is interesting to note that the divergence values associated with the two fertility categories defined here do not overlap more substantially.

The lack of available sequences for nuclear loci (relative to mitochondrial loci) limited our ability to test whether nuclear genes generally produced the same pattern as the mitochondrial loci across all our species pairs. Despite this limitation, we were able to identify four nuclear genes: Zinc finger Y-chromosomal protein (ZFY), Zinc finger X-chromosomal protein (ZFX), Growth Hormone Receptor (GHR), Cholinergic Receptor Nicotinic Alpha (CHRNA1), that had been sequenced in 10 primate pairs (Fig. S3) known to produce viable hybrid offspring [21]. We generated pairwise distances for each of these genes using the same methodology employed in the mitochondrial analyses. We then assigned each species pair to Category 1 or Category 2 based upon their CYTB divergence values within the original framework. In each case, though the order of the taxa based upon pairwise divergence values varied relative to the pattern generated using *CYTB* (owing to the significantly smaller divergence values in nuclear loci), for each locus the limited overlap in divergence values associated with the two categories was consistent with the mitochondrial assessment (Fig. S4).

In order to further substantiate both this correlation and the robustness of *CYTB* as a proxy for hybrid fertility, we tested the utility of this system for predicting fertility in a well-known hybrid system. To create hybrid pets, cat breeders have crossed domestic cats (*Felis catus*) with three species of wild felid: Jungle cats (*F. chaus),* Leopard cats (*Prionailurus bengalensis*), and Servals (*Leptailurus serval*) [27]. In all

cases, the first generation male hybrids are sterile. To regain fertility while maintaining some wild cat characteristics, breeders must backcross the F1 female offspring with male domestic cats to establish a breeding population of pets [27]. Given that backcrossing is required for all three crosses, our framework would firstly predict that the *CYTB* distances between all three pairs should be close to or greater than ~7.5% and that they should all fall into the Category 2 range. Secondly, the pairs with greater genetic distances should require more numerous backcrosses with domestic cats (halving the wild cat ancestry with each subsequent generation) before full fertility is restored and a breeding pet population is established.

Both of these predictions are borne out by the data (Fig 1, Table S3). All three pairs possess *CYTB* distances greater than or equal to 7.54% and the increasing molecular distances between the pairs correlate with an increase in the number of required backcross generations to regain fertility. Specifically, distances between Jungle cats, Leopard cats, Servals and domestic cats (7.54%, 10.94%, and 11.28% respectively) are consistent with both the observed minimum (2, 3, and 4 respectively) and average (3, 4, and 5, respectively) number of backcrosses with domestic cats required for hybrid males to acquire fertility [27]. These results are also consistent with an early hybrid experiment using guinea pigs in which hybrids between *Cavia fulgida* and *C. porcellus* (8.02% *CYTB* divergence) were able to regain male fertility after the third generation of backcrosses [28](Table S1).

Recent accidental hybrids in zoos also confirm the predictive power of this proxy. In 2006, the Copenhagen Zoo placed a domestic sow (*Sus scrofa domesticus*) in a pen with a male babirusa (*Babyrousa celebensis*) with the expectation that the two species were sufficiently evolutionarily divergent that they would be incapable of producing offspring. The *CYTB* divergence between the two species (12.89%), however, falls within the Category 2 range. Months later, five infertile piglets were born and though two died from maternally induced trauma, the other three (two males, and one female), all survived [29] (Table S1, Fig. S1). Historically, zoos have often accidentally produced hybrids offspring between distantly related species (Table S2), though the relative fertility of the F1s was rarely established.

Having approximated the *CYTB* threshold values within the framework, we then predicted the relative fertility of hybrids between pairs of other hominin lineages. To do so, we calculated the average pairwise divergence in *CYTB* sequences between AMH and three extinct hominin lineages: Neanderthals, Denisovans, and the ancient population from the Sima de los Huesos cave in Spain [29,30]. To avoid overestimating the genetic divergence resulting from the comparison of modern and extinct populations, we also generated distance values using the CYTB sequences derived solely from ancient AMH found in archaeological contexts.

The divergence values for each pairing between three *Homo* groups (Sima de los Huesos, Neanderthals, and AMH) all possess divergence values at the bottom of the Category 1 range (Fig 1, Fig. S1, Table S1). Interestingly, the divergence values of Denisovan-Neanderthal and Denisovan-AMH are the largest of the *Homo* pairings, and are consistent with the suggestion that Denisovans possess a mitochondrial lineage that may have introgressed from another source population [5].

In order to assess hybrid fertility within the hominin lineage more deeply, we calculated the pairwise *CYTB* divergence between humans and our closest living relatives: chimpanzees (*Pan troglodytes*) and bonobos (*P. paniscus*). Female chimpanzees inseminated with human sperm during a Soviet experiment in the 1920s failed to produce any offspring, and the reverse experiment did not progress beyond the planning stage [31]. Recent molecular clock assessments have suggested that AMH and chimps diverged ~5-6 Mya [32], well beyond both the two million year threshold purported by other studies as the upper limit to hybrid fertility [15,16], and the average time to speciation [33].

Interestingly, our analysis places the divergence values between AMH and chimps, and AMH and bonobos within Category 2 (Fig 1, Fig S1). Four other species pairs (including two of the wild cat and domestic cat pairs) possess divergence values equivalent to or greater than AMH, and bonobos (Table S1, Fig. S1). In all four cases, however, each of the pairs possesses an identical number of chromosomes.

Though karyotypic divergence is not a strict barrier to the production of hybrids (Table S1), AMH possess two fewer chromosomes than chimps and bonobos (Table S1).

Conclusions

When placed within the context of other mammalian species, ancient hominin lineages were likely not sufficiently divergent from each other to expect a biological impediment to the generation of fertile offspring. Although some studies have inferred a low level of introgression [33,34], a more recent study [35] concluded that there has not been any long-term removal of Neandertal DNA across the genome. In addition, the relative ease of hybridisation between hominin lineages is demonstrated by the fact that archaic populations interbred with AMH on at least four occasions [36].

The absence of a biological barrier shifts the focus toward cultural considerations and demographic factors to explain the lack of Neanderthal and Denisovan genomic contribution on the AMH X chromosome, and the lack of mitochondrial DNA from either species within AMH. Palaeoanthropologists disagree as to what differences in culture, behaviour and cognition existed between AMH and Neanderthals (*e.g.* [37,38]). Though these factors may have altered the sexual symmetry of the hybrids, they appear not to have precluded introgression in either direction [39].

This study demonstrates that genetic divergence can be used as a proxy to predict the relative sterility of hybrids resulting from matings between pairs of terrestrial mammalian species. Establishing which species pairs violate the predictions of the framework may lead to a better understanding of the process of speciation and the biological and sociocultural mechanisms responsible for hybrid sterility which is both fluid and governed by numerous mechanisms at multiple levels of biological organization. In addition, gene flow between species whose hybrids require backcrossing is possible (Table S1), as are asymmetries in gene flow in species pairs whose F1 offspring can produce fertile F2s.

We do not claim that *CYTB* plays a causative role in hybrid sterility (though recent studies have proposed that speciation may be mediated by mitonuclear interactions [40,41]), and the use of genetic divergence values as a proxy cannot be perfectly predictive. For example, under the Dobzhansky-Müller model, incompatibility can arise from as few as two mutations in each of the admixing populations irrespective of time since divergence, meaning that it would be possible for closely related populations to be incapable of generating fertile hybrids [40,42], though no such examples have yet been described.

The value of any proxy, however, is determined by both its predictive power and the ease of generating the proxy data. Publicly available mtDNA sequences from thousands of mammalian taxa already exist and calculating pairwise divergence values is inexpensive, simple and fast. As a result, genomic distances have substantial value as a means to predict the potential for any two mammalian species to produce viable offspring, and the relative degree of the hybrid's sterility. The discovery of additional extinct hominin populations that survived into the last 250,000 years, including *H. floresiensis* and *H. naledi*, has raised interest in understanding the limits to fertility and hybridization between extinct and extant *Homo* spp. If and when mitochondrial genomes from these samples can be obtained, the approach described here may provide an answer, even if nuclear genomic data are not obtainable.

Materials and Methods

Assessment of Hybrid Fertility and Rationale of Assignment into Categories

In order to ascertain if there was a relationship between genetic divergence and the fertility of hybrid offspring between species, we first collected published examples of species pairs that were capable of producing live offspring. We then split the hybrid pairings into two categories. Into Category 1 we placed eight species pairs that are capable of producing fertile F1 offspring of both sexes that can reproduce without backcrossing with a parent species. Though within these examples there have been observed asymmetries in gene flow and variation in fertility amongst the hybrids,

none of these examples requires (one or more generations of) backcrossing with a parent species for their offspring to regain fertility. More specifically, for an example to be listed in this category, we required evidence of captive breeding experiments that produced F2s. The evidence and rationale for placing each of these pairs into Category 1 is listed in Table S1 and the flow chart is depicted in Table S2.

The hybrid offspring of all of eight pairs of species in Category 2 either require one or more generations of female hybrids backcrossing with the male of a parent species to produce fertile offspring, or are completely infertile. For these pairs, we obtained evidence demonstrating no successful F2s from F1 hybrid couplings, an inability to produce offspring other than by backcrossing to a parent species, or other biological assessments (including a histological assessment of the testis from the hybrid males) that demonstrated complete infertility (Table S1).

For instance, in the case of hybrids between lions (*Panthera leo*) and tigers (*P. tigris*), there are numerous anecdotal reports regarding hybrid male sterility in ligers (male lion x female tiger) and the backcross hybrid offspring derived from females (e.g. [43]. Here, we provide histological evidence of sterility based on H&E stained testes from an adult male liger (Fig. S5 panels a and b), and an adult male tiliger (male tiger x female liger; panels c and d). Testes show clear seminiferous tubule degeneration, lined only with Sertoli cells in the liger, and tubule degeneration with germ cell arrest in the tiliger (Fig. S5 panels c, d).

Many additional live hybrid offspring have been reported in the literature than are included in Fig. 1 or in Fig. S1. The relative fertility of the hybrid offspring, however, has not been adequately assessed and we were therefore unable to confidently place them into either category. The framework and threshold values depicted in Fig. 1 allow us to predict the fertility of these offspring given the definitions described above and their placement into Category 1 or 2. These pairs are listed in Table S2 and their relative positions are depicted in Fig. S3.

Genetic Distance Calculation

Both *CYTB* sequences and full mitogenomes (excluding the control region) of multiple individuals of each species were collected from Genbank (Table S4) and aligned using Clustal Omega version 1.2.4 [44]. In order to ensure that none of the sequences was either mislabelled or were nuclear copies of mitochondrial genes, we constructed Neighbour-Joining trees using Geneious version 6.1.8 [45] and removed all individuals that did not fall into monophyletic clades of each species. We first used *pModelTest* version 1.04 [46] to determine the best model for the alignment of each set of sequences for both species. We then calculated pairwise distances between each species pair using RAXML version 8 [47] and FASTTree version 2.1 [48]. We also generated raw distance values (i.e. the proportion of sites that differ between each pair of sequences) using the Hamming distance method which sums the number of base pair differences, regardless of whether they are transitions or transversions, and divides that number by the sequence length.

The distances were generated from the *CYTB* (and nuclear gene) alignments for each set of species pairings in fasta file format using a python wrapper to wrap around the programs RAXML, FastTree, pModelTest, and a custom python script written to test Hamming distance of sequences making use of the distance module version 0.1.3 [49] and Biopython version 1.68 [50] modules. The script was written for use in version 2.7 of the Terminal application in an GNU/Linux environment, and the source code is included at the end of this Supplementary Information. To generate the Hamming distance calculations, the aligned sequences had to be the identical length (meaning all partial sequences were rejected). In addition, gaps in the published sequences were ignored across those loci and across each alignment even if a nucleotide was present at those loci in other sequences to minimise the artificial inflation of the divergence value differences.

Mann-Whitney test of statistical difference between *CYTB* distance in hybrid categories

The statistical significance of observed differences in mitochondrial genetic distance between groups with reduced hybrid compatibility and those with full compatibility,

was tested using the Mann-Whitney U test (p = 6.216e-4) implemented in the R-software package [51].

Figure Legends

Figure 1

A depiction of the correlation between *CYTB* divergence between terrestrial mammalian species pairs and the relative fertility of their hybrid offspring. In column A, the green circles represent species capable of producing fully fertile F1 offspring which can reproduce independently of their parent species (Category 1). Brown circles represent species pairs whose F1 offspring either require backcrossing with a parent species or are completely infertile (Category 2). The lighter green and brown shaded regions represent the range of divergence values of the two categories and, given their general lack of overlap, can be used to determine the relative fertility of hybrid offspring. Column B depicts the divergence between three wild felid species and domestic cats, as well as the minimum number of generations of backcrosses with domestic cats before full fertility of the hybrid is restored. The white circles in Column C depict the divergence between three ancient hominins and AMH, as well as the distances between AMH and chimpanzees and bonobos (in Category 2). The asterisks represent those pairs that include modern samples of AMH. The lack of an asterisk signifies that only sequences derived from archaeological AMH were used to compute the divergence values. All hominin pairs are positioned at the lowest (most fertile) end of Category 1, suggesting that their hybrid offspring are likely to have been fully fertile. Additional detail regarding the specific species pairs are listed in Fig. S1 and Table S1.

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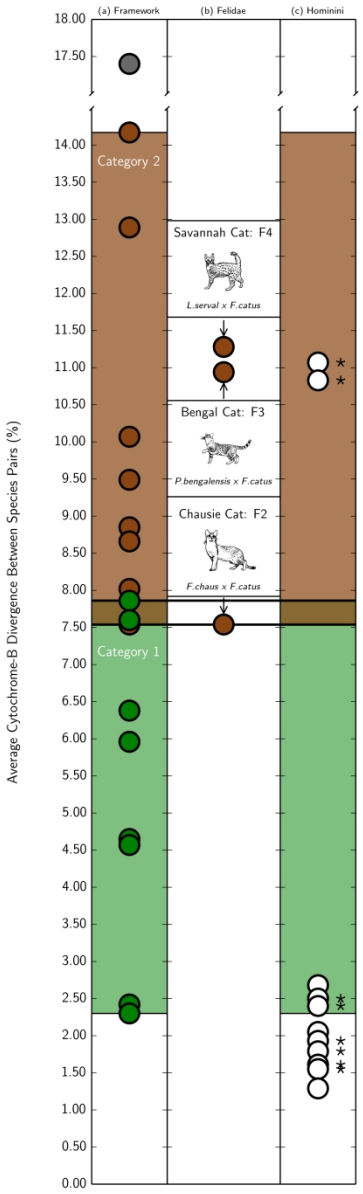
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