THE EVOLUTION, ECOLOGY AND GENETICS OF SEX DETERMINATION IN *MERCURIALIS ANNUA*

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ABSTRACT

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The allocation of resources to male or female progeny, or to male or female reproductive function more generally, is one of the most important life history decisions a sexually reproducing individual must ever make. Sex determination is thus a fundamental process, yet the mechanisms which control it are surprisingly diverse. In this thesis, I examine sex determination in the plant species *Mercurialis annua* L. (Euphorbiaceae). I assess the mechanism of sex determination operating in dioecious and androdioecious populations of *M. annua* and also investigate the conservation and evolution of sex-determining mechanisms across the annual mercury clade, the lineages of which display exceptional variation in sexual system.

First, using crosses, I establish that sex in dioecious *M. annua* is controlled by a single-locus genetic mechanism, consistent with recent work that identified a single male-linked DNA marker in the species. My search for new sex-linked genes revealed none, however, suggesting that *M. annua* possesses at most a small non-recombining region around sex-determining loci. Why many dioecious plants lack heteromorphic sex chromosomes is still poorly understood and I consider explanations for this. I extend my investigation by comparing genetic diversity between loci that differ in their linkage to the sex-determining locus. I find a single male-linked marker to possess significantly lower diversity than autosomal loci, but no difference in the diversity of partially sex-linked and non-sex-linked genes.

I also assess the conservation of a sex-linked marker among annual mercury lineages and conduct crosses between lineages to examine the conservation of sex determination. My findings indicate a conserved mechanism of single-locus genetic sex determination and I consider the role polyploidisation and hybridisation have played in sexual system evolution and the modification of sex-determining mechanisms in the clade. Finally, I assess the presence of environmental sex determination in androdioecious *M. annua*, concluding that although male frequency is not influenced by growing density, a degree of sexual lability exists in the lineage.

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The identification of new sex-linked genes in dioecious *Mercurialis annua* by segregation analysis of sequenced gene transcripts, presented in Chapter 3 of this thesis, was conducted in collaboration with Dr. Dmitry Filatov at the Department of Plant Sciences, University of Oxford. D. Filatov performed RNA extractions, transcriptome assembly and the segregation analysis using the program *Segregator*; I conducted crosses of plants, the verification of sex-linkage by manual low-throughput approach and the testing of putatively Y-linked genes in individuals from wild populations of dioecious *M. annua*.

In Chapter 4, sequencing of the DNA marker *OPB01-1562* in dioecious *M. annua* individuals from across the species' range was undertaken by Ian Turner as part of an undergraduate research project at the University of Oxford, under the supervision of Professor John Pannell and myself. I. Turner, J. Pannell and I jointly conceived and designed the study; I. Turner grew all plant material, performed DNA extractions, polymerase chain reaction (PCR) amplification and sequencing of *OPB01-1562* under my tutelage; I. Turner and I jointly assembled sequence data; and I analysed all data. The sequencing of all putatively pseudoautosomal and autosomal genes was conducted solely by myself.

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1. GENERAL INTRODUCTION

1.1 SEX DETERMINATION

Sex determination, defined here as the means by which the gender of a sexually reproducing individual is established, is a fundamental biological mechanism and one of the most important aspects of a species' biology (Bull, 1983). Following the evolution of sexual reproduction, mechanisms which regulate male and female sexual expression within an individual are essential in order to control the production of male and female progeny, or the investment of resources in male and female reproductive function more generally (sex allocation). With a finite quantity of resources and an often limited opportunity to reproduce, the effective allocation of resources is essential in order to maximise reproductive success and thus maximise an individual's fitness. For example, fitness will often depend directly on the number of males and females available to mate with, or how other hermaphrodites allocate resources to male and female function. Sex ratio selection is consequently thought to be a dominant driver in the evolution of sex-determining mechanisms (Bull, 1983). In addition to the regulation of reproductive function, sex-determining mechanisms can have implications for a species' genome and physiology. For example, selective pressures acting on sex-determining genes and genes associated with male and female fitness may culminate in the evolution of non-recombining, highly degenerate sex chromosomes (Charlesworth, 1991; 2002; Charlesworth *et al.*, 2005; Fraser & Heitman, 2005). Furthermore, the development of gender may also lead to the development of a myriad of secondary sexual characters (Lloyd & Webb, 1977; Dawson & Geber, 1998; Delph & Ashman, 2006; Bedhomme & Chippendale,

2007). Sex-determining mechanisms can hence be said to play a fundamental role in a species' biology.

Despite the pivotal role of sex-determining mechanisms, huge variation exists in the specific systems employed by individual species. Three broad classes of sex determination are generally recognised: genetic sex determination (GSD); environmental sex determination (ESD); and mixed genetic-environmental sex determination (Bull, 1983), although recent thinking emphasises the fact that both genetic and non-genetic factors are often involved in sex determination and that the supposed dichotomy between GSD and ESD is hence somewhat misleading (Uller & Helanterä, 2011). Nonetheless, a wide diversity of mechanisms exists along the continuum between GSD and ESD, with huge variation present in both the plant and animal kingdoms (Bull, 1983; Meagher, 1988; Kraak & Pen, 2002). Table 1.1 summarises some of the more common and noteworthy systems of sex determination reported across the natural world. Reasons for the wide variety of different mechanisms adopted by species are unclear, but it is likely to be at least partially due to numerous independent evolutionary origins of unisexuality and the subsequent evolution of novel systems of sex determination (Hodgkin, 1992; Tandurdzic & Banks, 2004).

Study of sex determination is interesting for a number of reasons. For example, given the fundamental importance of sex-determining mechanisms, it is surprising that such diversity in mechanisms exists between species and it is interesting to consider reasons for this pattern (e.g. Uller *et al.*, 2007; Pen *et al.*, 2010). As noted above, it is thought to be at least partially due to the independent evolution of

unisexuality in different taxa (Hodgkin, 1992; Tandurdzic & Banks, 2004), and thus it is interesting to compare the implications of the evolution of separate sexes for sex-determining mechanisms between species (Diggle *et al.*, 2011). It is equally interesting to consider the extent to which sex determination is conserved between lineages (Ferguson-Smith, 2007; Mrackova *et al.*, 2008; Williams & Carroll, 2009; Graves & Peichel, 2010), particularly in species in which closely related lineages display different sexual systems, which provide an opportunity to study the effects of transitions in sexual system on sex-determining mechanisms (e.g. Wolf *et al.*, 2001; Dorken & Barrett, 2004). Furthermore, given the implications of sex determination for physiology, study of sex-determining mechanisms is also important from a developmental perspective (Pinyopich *et al.*, 2003; Airoldi, 2010), with examination of the underlying molecular mechanisms useful in the study of genetic pathway evolution (Stothard & Pilgrim, 2003; Williams & Carroll, 2009; Diggle *et al.*, 2011; Gempe & Beye, 2011). Moreover, it is interesting to study the different conditions under which GSD and ESD may be favoured, and to study species in which mixed genetic-environmental systems of sex determination have been reported, especially given the prediction that the dichotomy between GSD and ESD is somewhat misleading (Baroiller *et al.*, 2009; Radder *et al.*, 2009; Uller & Helanterä, 2011). Study of sex chromosome evolution is also important for understanding the processes which drive the development of non-recombining, highly degenerate genomic regions (Charlesworth, 2002; Charlesworth *et al.*, 2005; Fraser & Heitman, 2005; Ellegren, 2011). Why, for example, have heteromorphic sex chromosomes evolved in some species but not others? It is likewise interesting to examine the evolutionary consequences of recombination suppression in sex chromosomes (Charlesworth, 1996; Bergero & Charlesworth, 2009), particularly with regard to

genetic diversity (Bachtrog & Charlesworth, 2002; Hellborg & Ellegren, 2004; Qiu *et al.*, 2010), the maintenance of gene integrity (Graves, 2006; Marais *et al.*, 2008; Chibalina & Filatov, 2011) and the adaptation of sex-linked genes (Rice, 1984; Charlesworth *et al.*, 1987; Vicoso & Charlesworth, 2006).

In this thesis, I examine sex determination in the plant species *Mercurialis annua* L. (Euphorbiaceae). The study of sex determination is particularly interesting in this species for a number of reasons, including: i) gender in dioecious *M. annua* has been proposed to be controlled by a rare multi-locus genetic system, unique amongst dioecious plant species (Louis, 1989); ii) the species does not possess heteromorphic sex chromosomes (Durand, 1963), indicating that chromosomes on which sex-determining loci are located may not have degenerated; iii) the species forms part of a larger clade of annual mercuries which displays great diversity in sexual system, and thus variation in mechanisms of sex determination; and iv) hexaploid androdioecious *M. annua* (populations in which males coexist with functional hermaphrodites) has been reported to possess a mixed genetic-environmental system of sex determination, where male frequency is influenced by growing density (Pannell, 1997a). *M. annua* is hence an ideal model system for examining many of the key questions surrounding the evolution and maintenance of sex-determining mechanisms. In the following sections of this chapter I introduce my study system and briefly outline my specific experimental investigations, with more detailed background and discussion of the relevant questions given in the chapters that follow.

Table 1.1 Definitions and examples of some of the more common and noteworthy mechanisms of sex determination reported in species across the natural world.

Mechanism	Genetic/ Environmental	Definition	Examples
XY (male heterogamety)	Genetic	Sex is determined by the inheritance of a single segregating genetic factor, with males (XY) heterozygous and females (XX) homozygous for the sex-determining region. Chromosomes carrying the sex-determining region may be homomorphic or heteromorphic. Gender may be determined by the presence of the Y factor (active-Y sex determination) or by the ratio of X chromosomes to autosomes (X:A sex determination).	Mammals (active-Y; Ford *et al.*, 1959; Jacobs & Strong, 1959; Vilain & McCabe, 1998) *Drosophila melanogaster* (X:A ratio; Bridges, 1925) *Silene latifolia* (active-Y; Westergaard, 1958; Nicolas *et al.*, 2005)
ZW (female heterogamety)	Genetic	As for male heterogamety, except that females (ZW) are heterozygous and males (ZZ) homozygous for the sex-determining region.	Birds (Tagaki & Sasaki, 1974) Lepidoptera (Traut *et al.*, 2008) *Fragaria virginiana* (Spigler *et al.*, 2008)
Polygenic	Genetic	Sex is determined by the inheritance of alleles at multiple loci, each of small effect.	*Xiphophorus helleri* (Kosswig, 1964) *Dicentrarchus labrax* (Vandeputte *et al.*, 2007)
Haplo-diploidy	Genetic	A system in which one sex, generally males, arise from unfertilized eggs and are haploid, whilst the other sex, generally females, arise from fertilized eggs and are diploid.	Hymenoptera (White, 1973)
Environmental sex determination	Environmental	Sex is determined at a point after fertilization according to the level of one or more environmental factors. An individual's genotype has no influence over its gender. A huge range of environmental cues may determine sexual development, including both biotic and abiotic factors.	Lizards, Turtles & Crocodilians (temperature-dependent sex determination; Janzen & Paukstis, 1991) Mermithids (resource availability; Petersen, 1972) *Ceratopteris richardii* (antheridiogen presence; Banks *et al.*, 1993)
Sexual lability	Mixed genetic-environmental	Sex is generally determined genetically, but sexual expression may be altered according to one or more environmental factors.	*Pleurodeles waltlii* (ZW with temperature influences; Houillion & Dournon, 1978) *Juniperus osteosperma* (water availability; Freeman *et al.*, 1981)

1.2 THE *MERCURIALIS ANNUA* STUDY SYSTEM

Mercurialis annua L. (Euphorbiaceae) is a ruderal, wind-pollinated, annual coloniser found throughout Europe and north Africa (Durand, 1963). Across the geographic range of the species, populations display variation in sexual system and ploidy levels. For example, all diploid ($2n = 16$) populations are dioecious and are widespread through eastern, central and western Europe. In contrast, polyploid lineages, which range in ploidy level from tetraploid to 12-ploid, are monoecious[1] or androdioecious (males coexisting with functional hermaphrodites) and have a much narrower geographic distribution. Specifically: tetraploid populations are exclusively monoecious and are restricted to southern Morocco; hexaploids are either monoecious or androdioecious and are found across the Iberian peninsula and north Africa; and octoploids and higher ploidy levels are monoecious and are restricted to Tunisia, Corsica and Sardinia (Figure 1.1; Durand, 1963; Durand & Durand, 1992). Together, these diploid and polyploid lineages of *M. annua* form the *Mercurialis annua* species complex.

The various lineages of the *M. annua* species complex are morphologically highly similar to one another. Males of dioecious and androdioecious populations are distinguishable by their distinct inflorescence morphology, with sessile staminate flowers arranged in spiral clusters along erect axillary peduncles. In females and monoecious individuals, pistillate and staminate flowers are usually subsessile and are borne on short pedicels in the leaf axils. Floral morphology in *M. annua* is highly variable, however, and female and monoecious individuals are known to

[1] Monoecious *M. annua* individuals may also be referred to as hermaphroditic or cosexual during the course of this thesis, in order to reflect both their functional gender and previous literature on the species and plant sexual systems in general (Lloyd, 1982; Pannell, 1995).

occasionally bear flowers on pedunculate inflorescences (Thomas, 1958; Durand, 1963; Durand & Durand, 1991; Pannell, 1997a).

In addition to the polyploid lineages of *M. annua* described above, two further species have been identified which, together with *M. annua*, are believed to form a clade of weedy annual mercuries. *Mercurialis huetii* Hanry is a diploid ($2n = 16$), dioecious species with a narrow geographic distribution in northeastern Spain and southern France. It is morphologically similar to, though distinguishable from, *M. annua* and is found in relatively undisturbed habitats, unlike *M. annua*, populations of which are generally found in highly disturbed, anthropogenic habitats (Durand, 1963). *Mercurialis canariensis* Obbard & S. A. Harris is a tetraploid ($4x = 32$), dioecious species endemic to the Canary Islands. It is likewise morphologically similar to *M. annua*, though distinguishable by the presence of male flower bracts and large stipules (Obbard *et al.*, 2006a).

Recent phylogenetic analyses indicate *M. huetii* and *M. canariensis*, together with diploid, tetraploid and hexaploid *M. annua*, form a single clade (Krähenbühl *et al.*, 2002; Obbard *et al.*, 2006b). Tetraploid *M. annua* is believed to be of autopolyploid origin, arising from duplication of the diploid *M. annua* genome. Hexaploid *M. annua* is hypothesised to be of allopolyploid origin, with evidence suggesting tetraploid *M. annua* to be the maternal and *M. huetii* the paternal progenitors. *M. huetii* itself is believed to be the sister species to diploid *M. annua*. *M. canariensis* is also thought to be of allopolyploid origin, with diploid or tetraploid *M. annua* as the maternal parent and an unknown species, potentially from outside the annual mercury clade, as the paternal parent (Figure 1.2; Obbard *et al.*, 2006b).

M. annua has been a model system for the study of sex determination for almost a century (Yampolsky, 1919; 1957; Gabe, 1939; Durand, 1963; Louis, 1989; Durand & Durand, 1991; Pannell, 1997a; Khadka *et al*., 2002; 2005). Studies have focused predominantly upon the dioecious lineage, in which both multi-locus and single-locus genetic systems of sex determination have been proposed (Yampolsky, 1919; Louis, 1989; Khadka *et al*., 2002; 2005). Cytogenetic studies have revealed no distinguishable heteromorphic sex chromosomes in the lineage (Durand, 1963). Sex determination in the androdioecious lineage has also drawn recent attention and is particularly interesting given the rarity of the mating system and theoretical predictions that androdioecy should be harder to evolve and maintain than dioecy or gynodioecy (Lloyd, 1975; Charlesworth & Charlesworth, 1978a; Charlesworth, 1984; Pannell, 2002), with recent evidence suggesting the lineage to possess a mixed genetic-environmental system of sex determination (Pannell, 1997a). How sex determination has evolved through the annual mercury clade following polyploidisation and hybridisation events remains unknown, however, and is one of the questions I seek to address in the present study.

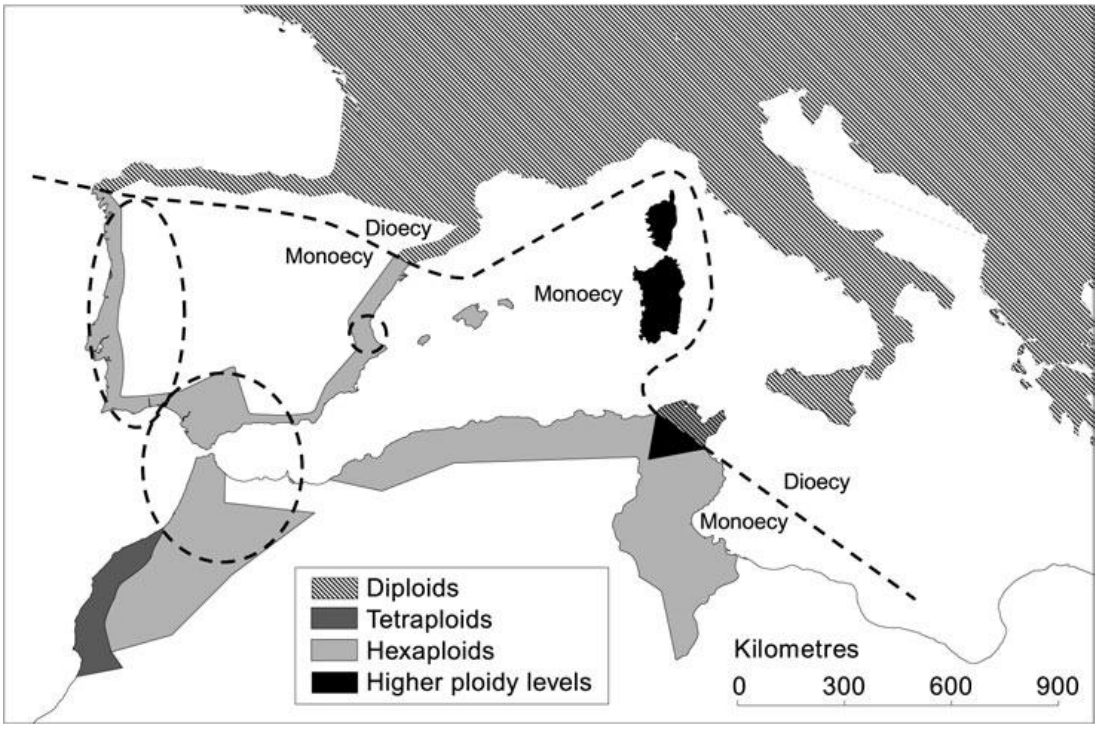


Figure 1.1 The distribution of the different ploidy levels and sexual systems of the *M. annua* species complex across Europe and north Africa. Dashed ellipses indicate the regions in which androdioecious populations have been reported. Map taken from Pannell *et al*. (2004) and originally modified from Durand (1963).

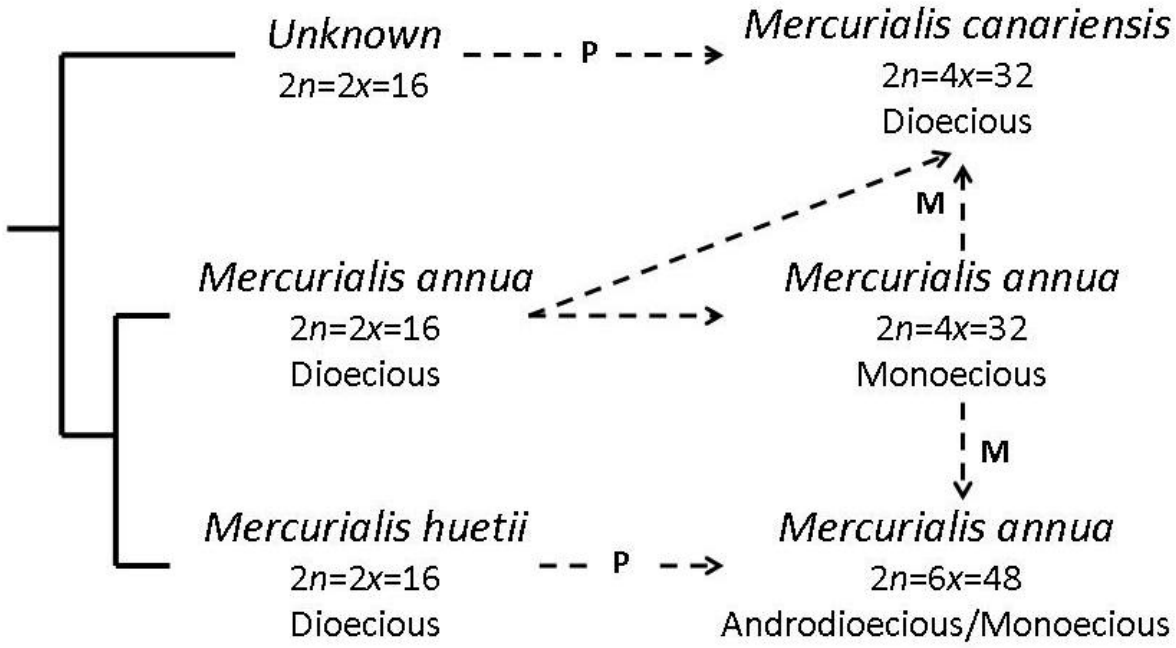


Figure 1.2 Hypothesised relationships between the annual lineages of *Mercurialis*. Filled lines indicate phylogenetic relationships between species and dashed arrows represent proposed hybridisation and/or polyploidisation events; M indicates proposed maternal parentage and P represents proposed paternal parentage. Figure based on Obbard *et al*. (2006b).

1.3 THE PRESENT STUDY

In this thesis, I seek to address several questions surrounding the evolution, ecology and genetics of sex determination in *M. annua*. Here, I briefly outline the collection of studies which comprise this thesis, with more detailed background and discussion of the specific questions addressed provided in the relevant chapters.

First, I examine the sex-determining mechanism of dioecious *M. annua* (Chapter 2), in which both multi-locus and single-locus genetic systems of sex determination have been proposed (Louis, 1989; Khadka *et al*., 2002; 2005). Multi-locus mechanisms of sex determination are very rare across the natural world and are generally considered to be evolutionarily unstable and vulnerable to displacement by single-factor sex determination (Bull, 1981; 1983; Rice, 1986; Uller *et al.*, 2007). Theory on the evolution of dioecy from hermaphroditism in plants also predicts the evolution of sex-determining mechanisms consisting of a single, rarely recombining, chromosomal region, rather than multi-locus or polygenic systems (Lewis, 1942; Charlesworth & Charlesworth, 1978a; 1980; Rice, 1987a; Charlesworth, 2002). Study of this potentially unique sex-determining mechanism in dioecious *M. annua* is therefore interesting given the rarity of its occurrence across both dioecious plants and other taxa. Through the use of sex ratio data obtained from half-sib and full-sib seed families, in conjunction with genetic data on the inheritance and segregation with gender of a putatively sex-linked DNA marker, I thus investigate the mechanism of sex determination operating in this lineage. I also consider the possibility of geographic variation in the species' sex-determining mechanism, which may account for the different models of sex determination put forward, and consequently sample plants for my study from across the species' range.

Furthermore, I examine the presence of family sex ratio variation in dioecious *M. annua*; mechanisms which bias sex ratios away from those expected purely from the Mendelian segregation of sex-determining genes are well documented in dioecious plants (Werren & Beukeboom, 1998; de Jong & Klinkhamer, 2002; Barrett et *al*., 2010) and I consider their occurrence in *M. annua*.

In Chapter 3, I examine the conservation of sex-determining mechanisms across the clade of annual mercuries using a sex-linked DNA marker. Dioecy is believed to be the ancestral state in the annual mercuries, and possibly in the genus *Mercurialis*, given the basality of dioecious species in molecular phylogenies of the genus combined with the fact that in the *M. annua* species complex dioecious populations are diploid whereas monoecious and androdioecious populations are polyploid (Durand & Durand, 1992; Krähenbühl *et al*., 2002; Obbard *et al*., 2006b). Assuming a shared ancestral basis for unisexuality, therefore, it is interesting to examine whether the specific sex-determining mechanism of lineages within the annual mercury clade has been conserved. This is particularly interesting in polyploid *M. annua* given the diversity in sexual systems they present and the hypothesised evolutionary history of the annual mercury clade (Obbard *et al*., 2006b). Identification of a conserved sex-linked marker in androdioecious *M. annua*, for example, may shed light on the evolution of this rare mating system. By means of polymerase chain reaction (PCR) amplification and DNA blot hybridisations of a putatively male-linked marker from dioecious *M. annua* (Chapter 2), I assess the presence and sex-linkage of this marker in other lineages of the annual mercury clade to assess the conservation of sex determination. This study is subsequently developed further as I search for new sex-linked markers in dioecious *M. annua* by a

segregation analysis of sequenced gene transcripts. By identifying multiple new putatively Y-linked genes in the dioecious lineage, I aim to test for their presence in other lineages of annual mercuries and thus potentially identify homologous sex-linked genes across the clade. However, the identification of numerous partially, but not fully, sex-linked genes raises the interesting scenario that recombination suppression, if it occurs, is restricted to a relatively small region around the sex-determining locus in dioecious *M. annua*. I discuss the full implications of these results in Chapter 3 and also compare sex chromosome evolution in *M. annua* to other taxa, including the plant species *Silene latifolia*, which possesses heteromorphic sex chromosomes (Westergaard, 1958).

Study of the chromosomes on which sex-determining loci are located in dioecious *M. annua* is extended in Chapter 4 with a comparison of the levels of genetic diversity in partially sex-linked, fully sex-linked and autosomal loci in the species. Theory predicts genetic diversity to be lower in Y-linked loci relative to X-linked and autosomal loci as a result of processes which reduce the effective population size of non-recombining chromosomal regions (Charlesworth & Charlesworth, 2000). In contrast, diversity is predicted to be elevated between partially X- and Y-linked loci (loci located on the same chromosome pair as genes for sex determination, but in a region in which recombination is not suppressed) close to the non-recombining region, largely as a result of sexually antagonistic selection (Kirkpatrick *et al.*, 2010; Otto *et al.*, 2011). Using partially sex-linked and putatively autosomal genes identified from the segregation analysis undertaken in Chapter 3, in addition to a putatively Y-linked DNA marker identified in the lineage (Chapter 2), I therefore test these predictions in dioecious *M. annua*. Study of the

implications for genetic diversity of the evolution of sex-determining genes is especially interesting in dioecious *M. annua* given the apparent lack of degeneracy in the chromosomes on which sex-determining loci are located (Durand, 1963) and the inference that recombination suppression is restricted to a relatively small region around the sex-determining loci (Chapter 3), and I compare the results obtained with those from systems with heteromorphic sex chromosomes, including *S. latifolia*.

In Chapter 5, I examine the direct effects of polyploidisation and hybridisation on sex determination and sex expression in the annual mercury clade. Polyploidisation is believed to have played a major role in plant evolution (Ramsey & Schemske, 2002), is known to disrupt sex-determining mechanisms (Muller, 1925; Westergaard, 1958) and has been implicated in the evolution of sexual system transitions (Pannell *et al.*, 2004). As outlined in section 1.2, evidence indicates both polyploidisation and hybridisation to have played key roles in the evolution of the annual mercury clade (Obbard *et al*., 2006b). The variation in sexual system with ploidy in the clade thus presents an opportunity to explore how these processes affect sex-determining mechanisms. The occurrence of androdioecy in populations of hexaploid *M. annua* is particularly interesting and I consider how polyploidisation and hybridisation may have been involved in the production of this rare mating system, as well as in the evolution of functional hermaphroditism (monoecy). Through the generation of colchicine-induced neo-polyploids and hybrid progeny from crosses between various lineages of the annual mercury clade, I thus examine the role of these processes in shaping the sex-determining mechanisms of annual mercuries. I also examine the gender and sex expression of hybrids produced in order to assess the conservation of sex-determining mechanisms amongst annual

mercuries and the functionality of sex-determining genes in different lineages. Furthermore, I perform a comparative morphological analysis of hybrid plants and their parent lineages to examine the effects of hybridisation on plant morphology.

Finally, in Chapter 6 I investigate the presence of environmental sex determination (ESD) in hexaploid androdioecious *M. annua*. ESD is predicted to evolve in a species when environmental conditions differentially influence male and female fitness and when individuals have little control over which environment they will experience (Charnov & Bull, 1977). Given the sessile nature of plants, we might therefore expect ESD to be relatively common amongst plant species, and this is perhaps reflected in the fact that many plant species display a degree of sexual lability (Korpelainen, 1998). Hexaploid androdioecious *M. annua* has been reported to possess a mixed genetic-environmental system of sex determination, with growing density influencing male frequency and sex allocation (Pannell, 1997a). I examine the role of growing density in determining gender and discuss the selective advantages of an environmental component to sex determination in this lineage, particularly given its colonising life history. I also consider alternative theories to account for the reported relationship between male frequency and density, including the possibility of geographic variation in the capacity for density-dependent gender choice, since other studies have failed to detect any environmental influence on sex ratios in the lineage.

In Chapter 7 I discuss my general findings and wider relevance of this work.

2. SEX DETERMINATION IN DIOECIOUS *MERCURIALIS ANNUA*

2.1 INTRODUCTION

The mechanisms of sex determination found in plant species are extremely diverse (Meagher, 1988; Tandurdzic & Banks, 2004; Ming *et al.*, 2011). Approximately 28 species of land plants have been identified as possessing heteromorphic sex chromosomes[2] (Ming *et al*., 2011), of which the majority employ an active-Y system of sex determination, analogous to that of mammals. Examples include the cycad *Cycas revoluta* (Segawa *et al*., 1971) and the angiosperms *Cannabis sativa* (Sakamoto *et al*., 1998) and *Silene latifolia* (Westergaard, 1958; Nicolas *et al.*, 2005). In contrast, the gymnosperm *Gingko biloba* possesses a ZW system of sex determination where females are the heterogametic sex (Chen *et al*., 1987; Lan *et al.*, 2008), whilst in other species with heteromorphic sex chromosomes, gender is determined by the ratio of X chromosomes to autosomes, such as in *Humulus japonicus* (Jacobsen, 1957; Grabowska-Joachimiak *et al.*, 2011) and *Rumex acetosa* (Ono, 1935; Navajas-Pérez *et al.*, 2005). Furthermore, around 20 species have been reported to possess homomorphic sex chromosomes, in which gender is controlled by a single, or several very tightly linked, loci. *Asparagus officinalis* (Loptien, 1979; Telgmann-Rauber *et al.*, 2007), *Sagittaria latifolia* (Dorken & Barrett, 2004)

[2] It is worth noting that the term 'sex chromosome' is used widely in literature on plant sex determination, yet its meaning has become somewhat confused. Traditionally, the term was used to describe only heteromorphic, degenerate sex chromosomes that could be identified cytologically (Westergaard, 1958; Bull, 1983). More recently, however, the term has been used to refer to chromosomes in single-locus or single-factor systems on which loci controlling sex determination are located, regardless of the extent of dimorphism (e.g. Jamilena *et al.*, 2008; Ming *et al*., 2011). In this thesis, I endeavour to clarify and distinguish between these scenarios at all times.

and *Spinacia oleracea* (Khattak *et al.*, 2006; Lan *et al*., 2006) are three such examples, with males being the heterogametic sex in each of these cases. In contrast, *Fragaria virginiana* (Spigler *et al.*, 2008) and *Populus trichocarpa* (Yin *et al*., 2008) have homomorphic sex chromosomes with female heterogamety. Environmental sex determination is also found in plant species, notably amongst homosporous ferns such as *Ceratopteris richardii* and *Woodwardia radicans* in which gametophytes develop as males only in the presence of antheridiogen, a hormone secreted into the soil by hermaphrodites (and females in the case of *W. radicans*; Banks *et al*., 1993; Korpelainen, 1998; Quintanilla *et al*., 2007). Sex in dioecious *Mercurialis annua*, however, has been proposed to be controlled by an alternative mechanism: a multi-locus genetic system unique among plant species in which three unlinked genes interact to determine gender (Louis, 1989).

Under the multi-locus model of sex determination in dioecious *M. annua*, three independently segregating, diallelic, nuclear loci, *A, B1* and *B2* (case indicates dominance), have been proposed to control reproductive organogenesis (Louis, 1989). Males develop in the presence of two complementary alleles, *A*, and either one of the *B* genes, whilst the genotypes *a*/*a* and *b1*/*b1*, *b2*/*b2* are female. This multi-locus mechanism was proposed following the identification of differences between males in their resistance to feminization by the exogenous application of cytokinins. These differences in 'male strength' were hypothesised to be controlled by the *B* genes: *B1* associated with *B2* confers complete resistance to feminization; *B1* alone gives intermediate resistance; whilst *B2* alone gives low resistance. Cytogenetic studies have also indicated that the species does not possess heteromorphic sex chromosomes (Durand, 1963).

This unusual multi-locus model of sex determination has entered the secondary literature (Dellaporta & Calderon-Urrea, 1993; Grant *et al*.; 1994; Ainsworth, 2000; Janousek & Mrackova, 2010), yet its maintenance is not well understood. Multi-locus and polygenic (when many loci of small effect determine gender) systems of sex determination are generally considered to be evolutionarily unstable and vulnerable to displacement by 'monogenic' sex determination (Bull, 1981; 1983; Rice, 1986; Uller *et al.*, 2007). For example, polygenic sex determination may be displaced if it is poorly canalized (when sex determination is also influenced by environmental factors; Bull, 1981; 1983), if a single, major sex-determining gene arises and either pleiotropically increases fitness itself or is linked to a selectively advantageous gene (Rice, 1986), or as a result of intergenomic conflict between parents and offspring (Uller *et al.*, 2007). Multi-locus or polygenic sex determination may be an intermediate state in the evolution of a species' sex-determining mechanism, perhaps following the invasion of a new sex-determining gene (e.g. as suggested for fish; Kirpichnikov, 1981; Bull, 1983), but are thought unlikely to persist over evolutionary time (Rice, 1986). This may explain why multi-locus and polygenic systems are rare across the natural world, being reported in only a handful of species, including the swordtail fish *Xiphophorus helleri* (Kosswig, 1964), the European sea bass *Dicentrarchus labrax* (Vandeputte *et al.*, 2007) and the housefly *Musca domestica* (Dübendorfer *et al.*, 2002; Kozielska *et al.*, 2006).

M. annua appears to be the only dioecious plant species for which a multi-locus system of sex determination has been proposed. Here, because the three loci (*A, B1*

and *B2*) are equivalent and each has the capacity to determine gender on its own, allele frequencies are theoretically free to drift until fixation is reached at two of the three loci, with the third, polymorphic, locus becoming the single sex-determining gene (Obbard, 2004). It would thus seem that some unknown process must be maintaining polymorphism at each of the three loci, if indeed the three-locus model is correct. It should be noted that polygenic systems have been proposed for the gynodioecious species *Plantago coronopus*, *Silene vulgaris* and *Thymus vulgaris*, in which cytoplasmic male-sterility genes and nuclear restorers interact to control male reproductive function and thus gender (Ehlers *et al.*, 2005). In these cases, however, mitochondrial sterility genes are exclusively maternally inherited and may be carried by, and thus inherited through, both sexual morphs (females and hermaphrodites), a scenario not present in dioecious species in which each sexual phenotype is, by definition, unisexual.

The multi-locus sex-determining mechanism of dioecious *M. annua* is also unusual when one considers the theoretical pathways by which dioecy is proposed to evolve from hermaphroditism in plants. It is widely accepted that unisexuality evolves as a consequence of selection for the benefits of inbreeding avoidance and/or sexual specialisation (Lloyd, 1975; Charlesworth & Charlesworth, 1978a; 1978b; Freeman *et al*., 1997). Two major evolutionary pathways from hermaphroditism to dioecy are generally recognised: the gynodioecy pathway, in which intermediate populations contain females and hermaphrodites (Charlesworth & Charlesworth, 1978a), and the monoecy-paradioecy pathway, in which intermediate populations display quantitative variation in male and female fertility (Lloyd, 1975; 1980; Charlesworth & Charlesworth, 1978b). As it is not thought possible for separate sexes to evolve

from hermaphroditism in a single mutational step (notwithstanding the extremely improbable occurrence of a mutation whose heterozygotes display only one sexual function and homozygotes the other; Lloyd, 1974; Charlesworth, 2002), both of these pathways to dioecy involve the establishment of two types of gene: those suppressing male reproductive function (male-sterility genes) and those suppressing female reproductive function (female-sterility genes; Charlesworth & Charlesworth, 1978a).

Sterility mutations are thought to become established in different ways along each of the two pathways. Under the monoecy-paradioecy pathway, the fixation of multiple mutations with small effects is proposed to cause a gradual shift in the gender of individuals (Lloyd, 1975; Charlesworth & Charlesworth, 1978b). In contrast, the gynodioecy pathway involves the initial establishment of a complete male-sterility mutation (giving rise to a gynodioecious population), followed by subsequent selection for female-sterility mutations that increase male allocation in the remaining hermaphrodites (Charlesworth & Charlesworth, 1978a). Theory predicts that there will be strong selection pressure for these two types of sterility mutation to become genetically linked, regardless of the evolutionary pathway involved (Charlesworth & Charlesworth, 1978a; 1980; Rice, 1984). This is because the independent segregation of male- and female-sterility mutations would give rise to both hermaphrodites, which have neither sterility factor, and neuter individuals, which are sterile for both sexual functions (Lewis, 1942; Charlesworth & Charlesworth, 1980; Rice, 1987a; Charlesworth, 2002). Selection for tight linkage between sterility mutations is thus predicted to lead to the evolution of single-factor sex-determining

systems in plants, with gender effectively controlled by the segregation of a single genetic factor possessing either male- or female-determining gene combinations.

In addition to ever-tighter linkage between loci controlling reproductive function, selection under antagonistic pleiotropy (when sex-determining alleles are beneficial in one sex, but not in the other) may also lead to suppressed recombination in this chromosomal region in order to prevent the break up of gene combinations conferring complementary sterility and functionality in male and female reproductive traits (Rice, 1987a; 1996; Charlesworth, 2002). Moreover, once males and females have become established, further sexually antagonistic genes may accumulate in the same chromosomal region, leading to the expansion of suppressed recombination along the length of the sex chromosome pair (Fisher, 1931; Rice, 1987a). For example, mutations beneficial to males but detrimental to female fitness are more likely to invade a population if linked to the male-determining genetic region than if located on autosomes, and thus the non-recombining region of sex chromosomes is predicted to expand to accommodate such genes (Charlesworth & Charlesworth, 1980; Rice, 1984; 1987a; 1996; Jordan & Charlesworth, 2012). Such recombination suppression may cause chromosomes to degenerate, as population level processes reduce the effective population size of the non-recombining region and the efficacy of selection, leading to the accumulation of transposable genetic elements, duplication of genome fragments, and a loss of genetic diversity and gene integrity, as observed in mammalian Y chromosomes (see section 4.1 for further explanation of these processes; Charlesworth & Charlesworth, 2000; Gerrard & Filatov, 2005; Ming *et al.*, 2011). These processes may thus culminate in the development of heteromorphic sex chromosomes, although this scenario is rare in

dioecious plants, being described in only 28 species to date (Jamilena *et al.*, 2008; Ming *et al.*, 2011).

Reasons for the rarity of heteromorphic sex chromosomes in plant species remain unclear (Charlesworth, 2002; 2008). Possible explanations include the relatively recent origin of dioecy in some plants (with there subsequently having been insufficient time for recombination suppression to evolve across a large region of the sex chromosome pair) or due to a lack of selection for sexual dimorphism in plants, which may limit the fitness advantages of linking sexually antagonistic alleles to the sex-determining locus (Charlesworth, 2008; Charlesworth & Mank, 2010). In cold-blooded vertebrates, most species of which possess homomorphic sex chromosomes (Devlin & Nagahama, 2002; Eggert, 2004), the lack of chromosome degeneracy has been proposed to be the result of either a high turnover in master sex-determining genes (analogous to the recent origin of dioecy theory in plants in that both involve the recent evolution of a sex-determining mechanism, with subsequent insufficient time for sex chromosomes to decay; Schartl, 2004; Volff *et al.*, 2007), or occasional recombination between sex-determining chromosomes (Perrin, 2009; Grossen *et al.*, 2012). Sex-reversal, common in many cold-blooded vertebrates (e.g. Baroiller *et al.*, 2009), has been suggested as a means by which recombination between sex chromosomes may occur (Perrin, 2009), as recombination patterns often depend on phenotypic rather than genotypic sex (e.g. Lynn *et al.*, 2005; Matsuba *et al.*, 2010). Given the lability in the sex expression of many plant species (Korpelainen, 1998), it is therefore possible that a similar process operates in dioecious plants to maintain sex chromosome homomorphism, although this has not been documented to date and does not explain why many dioecious plants lack large non-recombining regions in

the first place. Why heteromorphic sex chromosomes have evolved in some dioecious plant species but not others thus remains a major puzzle in plant and evolutionary biology and is discussed further in subsequent chapters of this thesis.

The theory outlined above therefore predicts the development of sex-determining mechanisms consisting of a single, rarely recombining, chromosomal region following the evolution of dioecy from hermaphroditism. Indeed, this fundamental similarity is observed in almost all dioecious species in which genetic mechanisms of sex determination have been studied, and different stages in the evolution of single-factor sex-determining mechanisms can be observed in different dioecious species (Ming *et al.*, 2011). For example, *F. virginiana* is thought to be at the earliest stage, with male- and female-sterility mutations tightly linked on a chromosome pair, but a low frequency of hermaphrodites and neuter individuals evidence that some recombination still occurs (Spigler *et al.*, 2008). In *A. officinalis*, recombination is believed to be suppressed around the sex-determining loci, but this region is very small (Telgmann-Rauber *et al.*, 2007), whilst in *Carica papaya* recombination suppression has expanded, with some chromosome degeneration, although chromosomes still appear homomorphic at the cytological level (Liu *et al.*, 2004). Finally, in *S. latifolia* recombination is suppressed over much of the length of the sex chromosome pair, with clearly identifiable heteromorphic sex chromosomes and evidence of genetic degeneration (Westergaard, 1958; Nicolas *et al.*, 2005; Marais *et al.*, 2008), whilst in *R. acetosa* the Y chromosome has been completely lost (Ono, 1935; Navajas-Pérez *et al.*, 2005). The multi-locus model of *M. annua* is therefore something of an exception, and no theoretical model has been put forward

to explain how a multi-locus or polygenic system of sex determination involving unlinked genes may arise and be maintained in a dioecious plant species.

Since the publication of the three-locus model of sex determination in dioecious *M. annua*, a recent study provides evidence for a simpler, one-locus model. In particular, Khadka *et al*. (2002) have identified a nuclear DNA (SCAR) marker, *OPB01-1562*, that co-segregates exclusively with male sex in the species. Given that a key feature of the three-locus model is that none of the alleles of the three loci are exclusively linked to a single sex, these two models are clearly in disagreement with one another. The work of Khadka *et al*. (2002; 2005) tested for the presence of *OPB01-1562* in individuals predominantly from Belgian populations of dioecious *M. annua*, with only 10 individuals of each sex tested from French and Israeli populations, whereas the three-locus model was based on crosses of genotypes collected from a single French population (Louis, 1989). Since dioecious *M. annua* is believed to have undergone postglacial range expansion from a refugium in the eastern Mediterranean and exhibits a geographic gradient in genetic diversity and allelic richness (Obbard *et al.*, 2006c), it is possible that geographic variation exists in the species' sex-determining mechanism. Specifically, allele frequencies of each of the genes of the three-locus model may vary between populations, with gender being controlled by a single locus in some populations if fixation is reached at two of the three loci, but the specific locus determining sex differing between populations. In order to comprehensively assess sex determination in the species, I here investigate the sex-determining mechanism of dioecious *M. annua* based on a much larger sample of plants from across the species' geographic range between the eastern Mediterranean and western Europe.

My analysis of sex determination in dioecious *M. annua* takes two approaches. First, I conduct a classic analysis of seed family sex ratios, analysing both half-sib families from wild female plants and full-sib families from controlled crosses. Analyses of sex ratios from controlled crosses have been used widely to infer systems of sex determination in dioecious plants (e.g. Westergaard, 1958; Wolf *et al.*, 2001; Dorken & Barrett, 2004; Glawe & de Jong, 2008), but they can be confounded by mechanisms that bias sex ratios away from those expected purely from the Mendelian segregation of sex-determining genes (Werren & Beukeboom, 1998; de Jong & Klinkhamer, 2002; Barrett et *al*., 2010), including: certation, the differential performance of male- versus female-determining pollen grains during pollen competition and pollen tube growth (Correns, 1922); segregation distortion by X-linked or cytoplasmic factors (Taylor & Ingvarsson, 2003); and differential germination and survivorship between the sexes (Lloyd, 1974; Allen & Antos, 1993; Lyons *et al*., 1995). These mechanisms are well documented in dioecious plants and can operate in spite of strict genetic sex-determining systems. For example, in *S. latifolia* sex ratios have been observed to range from 16.9% to 84.2% males (Taylor, 1994a), despite the presence of sex chromosomes. I thus also examine the inheritance and segregation with gender of the putatively sex-linked DNA marker *OPB01-1562* in controlled crosses, expanding substantially on the sampling of Khadka *et al*. (2002; 2005).

2.2 MATERIALS AND METHODS

2.2.1 Field-collected, half-sib seed family sex ratios

Seeds were collected from 48 female plants of dioecious *M. annua* from wild populations across the species' geographic range. Specifically, 12 seed families were collected from Israeli populations, 11 from UK populations, and 25 from Spanish populations (see Appendix 9.1 for full details of population locations). These populations were selected as they represent the most easterly and westerly edges of the species' distribution, thus incorporating the extremities of the observed longitudinal gradient in genetic diversity and allelic richness in the species (Obbard *et al*., 2006c) and therefore encompassing any spatial variation in the species' sex-determining mechanism. The seeds of each family were sown in soil-based compost in separate seed trays under standard glasshouse conditions and, upon flowering, all individuals were scored for sex to obtain family sex ratios.

2.2.2 Full-sib seed family sex ratios from controlled crosses

Seeds for crosses were collected from three wild populations of dioecious *M. annua*, again selected to represent the species' geographic range, specifically: HaGoshrim (Israel; Appendix 9.1), Sestri Levante (Italy) and Tarragona (Spain). Seeds from each population were collected from 20-30 large female plants and mixed thoroughly to create bulk seed stocks. In spring 2009, approximately 400 seeds from each population were sown in soil-based compost in seed trays. Following germination and flowering, all individuals were scored for sex to estimate population sex ratios. One male and four female plants from each population were then selected at random and each transplanted into an individual 15 cm diameter pot of soil-based compost. All plants were grown in the same glasshouse under standard conditions.

Once the transplants were established, crosses between the plants were undertaken. Each of the 12 female plants were crossed with the male individual from each of the three populations, thus generating three full-sib seed families per mother, each sired by a different father. Both within- and between-population crosses were thus undertaken to examine spatial variation in the species' sex-determining mechanism. To conduct crosses, a mating array was created in a field outside Wytham Woods, Oxford, UK, in which the 12 female plants were randomly positioned around a central pot containing the male plant from HaGoshrim. The pots were watered and maintained for a period of eight weeks to allow open pollination of the female plants by the male. To prevent pollen contamination from wild plants, the array was established a minimum of 50 m from all other *Mercurialis* plants. Female plants have been found to be strongly pollen-limited over distances exceeding 15 m (Hesse & Pannell, 2011a), and with the presence of a local male in the array, an exclusion zone of 50 m was deemed to be sufficient.

After eight weeks' growth, seeds from all 12 female plants were collected and the females cut back to just above the basal node. The male plant from HaGoshrim was then replaced with the male individual from Tarragona and the plants left for another eight weeks to allow crossing of the females with the second male plant. Slow-release fertiliser was applied to pots containing female plants to ensure they re-sprouted and were not resource-limited for further crosses. Finally, the above procedure was repeated and the third male plant (from Sestri Levante) positioned in the array to be crossed with the female plants. The 36 seed families obtained were subsequently sown in soil-based compost in separate seed trays under standard

glasshouse conditions. Upon flowering, all individuals were scored for sex to obtain a sex ratio for each cross.

Following the analysis of family sex ratios from these 36 crosses, eight further crosses were undertaken in summer 2010 using progeny from a seed family which was found to have a sex ratio that deviated significantly from 1:1. Four female progeny were crossed with each of two male progeny from the family using the same method as outlined above.

2.2.3 Data analysis

The sex ratios (number of males/total number of individuals) of all half-sib and full-sib seed families, plus wild populations, were tested for significant deviations from a 1:1 sex ratio using replicated goodness-of-fit tests (*G*-tests; Sokal & Rohlf, 1995). This approach allowed the total variation between expected and observed sex ratios (G_{total}) to be partitioned into that due to heterogeneity among individual seed families (G_{het}) and that due to a bias in the overall sex ratio away from 1:1 (G_{pooled}). Under a single-locus mechanism of sex determination one would expect to obtain a 1:1 sex ratio from all seed families (assuming no other mechanisms to bias sex ratios are in operation), whereas under the three-locus model one would expect significant departures from 1:1 in some seed families and, potentially, in the overall sex ratio, depending upon the specific alleles possessed by the mother and father at each of the *A*, *B1* and *B2* loci. The Dunn-Šidák method was used to control the Type I error rate and account for multiple tests when testing the sex ratios of individual seed families for deviations from 1:1 (Sokal & Rohlf, 1995).

In addition to *G*-tests, a generalized linear model (GLM) was used to test whether the sex ratios of field-collected half-sib seed families were influenced by their country of origin (to examine whether geographic variation exists in the species' sex-determining mechanism). Family sex ratio (number of males/total number of individuals) was used as the response variable, with country of origin fitted as a fixed factor, specifying a binomial error structure (full model: FAMILY SEX RATIO = COUNTRY).

A generalized linear mixed effects model (GLMM) was also used to analyse the sex ratios of full-sib seed families from controlled crosses, specifically: whether specific mothers or fathers used in crosses influenced sex ratios; whether the population of origin of mother plants influenced sex ratios; and whether the sex ratios from crosses involving parents from different combinations of populations differed significantly (i.e. whether there was a significant father x mother's population of origin interaction; as only one father was sampled per population, I was unable to differentiate between population- and individual-level paternal effects on sex ratios). Family sex ratio (number of males/total number of individuals) was again used as the response variable, with the mother's population of origin fitted as a fixed factor, the father's identity fitted as a random factor and the mother's identity fitted as a random factor nested within the mother's population of origin, similarly specifying a binomial error structure (full model: FAMILY SEX RATIO = MOTHERPOP | FATHER + MOTHER(MOTHERPOP)). All analyses were conducted in *R*, version 2.8.0 (http://www.r-project.org).

2.2.4 Inheritance of *OPB01-1562* and segregation with gender

Genomic DNA was extracted from fresh and dried leaf material using a modified cetyltrimethylammonium bromide (CTAB) procedure (Doyle & Doyle, 1987). Ground leaves were incubated in 2X CTAB at 65 °C, before being purified with two chloroform:isoamyl alcohol (24:1) extractions. Following precipitation using propan-2-ol at -20 °C, samples were washed in 70% ethanol, dried, and resuspended in Tris-EDTA (TE) buffer. Samples were stored at -20 °C until required.

DNA was extracted from all 15 parent plants used in crosses, plus four male and four female progeny selected at random from each of the 36 seed families obtained. All individuals were assessed for the presence of the DNA (SCAR) marker *OPB01-1562* and for a 766 bp control marker (believed to be from the same locus as *OPB01-1562* and likewise identified by Khadka *et al.*; 2002; 2005) by polymerase chain reaction (PCR) amplification with primer pairs B1F01/B1R01 and B1F01/B1R06 respectively (Khadka *et al.*, 2002). The reagents for all PCR reactions were as follows: 1X PCR buffer (supplied by Yorkshire Bioscience); 100 μM four dNTPs mix; 2 mM $MgCl_2$; 0.16 μM of each primer; 1.0 units *Taq* DNA polymerase (Yorkshire Bioscience); and 10 ng DNA in 25 μL volume. PCR amplification conditions were as follows: 1 cycle of 94 °C, 90 sec; 40 cycles of 94 °C, 30 sec, 58 °C, 30 sec, 72 °C, 90 sec; and a final cycle of 72 °C, 5 min. PCR products were visualized by ethidium bromide staining after electrophoresis on 1.5% agarose gels.

2.3 RESULTS

2.3.1 Sex ratio variation among field-collected half-sib seed families

Summed across all half-sib families, dioecious *M. annua* showed no evidence of deviation from a 1:1 sex ratio ($G_{\text{pooled }1} = 0.04$, $p = 0.836$). Significant heterogeneity was, however, detected among families ($G_{\text{het }47} = 77.8$, $p = 0.003$). Case-by-case comparisons using the Dunn-Šidák method for multiple tests identified one family that showed a significantly male-biased sex ratio (seed family 34a1, sex ratio = 0.724, $G_1 = 12.1$, $p = 0.024$; Table 2.1; Figure 2.1) and one that showed a marginally significant female-biased sex ratio (seed family BS10, sex ratio = 0.387, $G_1 = 9.76$, $p = 0.082$). Omitting these two seed families from the analysis greatly reduced the heterogeneity in the data, which was no longer significant ($G_{\text{het }45} = 55.7$, $p = 0.131$). Half-sib family sex ratios were not significantly influenced by their country of origin ($\chi^2_2 = 4.43$, $p = 0.109$).

2.3.2 Sex ratio variation among full-sib seed families from controlled crosses

None of the three wild populations from which individuals were sampled for crosses showed any deviation from a 1:1 sex ratio (Table 2.2). There was also no significant deviation from 1:1 in the overall sex ratio across all 36 full-sib seed families from controlled crosses ($G_{\text{pooled }1} = 1.33$, $p = 0.248$). However, like the field-collected half-sib seed families, there was significant heterogeneity among the 36 full-sib families ($G_{\text{het }35} = 59.8$, $p = 0.006$). This was due largely to a single seed family with a highly female-biased sex ratio (cross SpainG2 x IsraelM2; sex ratio = 0.376, $G_1 = 19.3$, $p < 0.001$; Dunn-Šidák method for multiple tests; Table 2.3; Figure 2.2). When this family was removed from the analysis, significant heterogeneity was no longer detected ($G_{\text{het }34} = 37.9$, $p = 0.298$), although the overall sex ratio across all

families became marginally significantly male-biased (sex ratio = 0.511, $G_{\text{pooled } 1}$ = 4.02, p = 0.045). However, eight further crosses using four female and two male progeny from this significantly biased family showed no significant deviation from a 1:1 sex ratio ($G_{\text{total } 8}$ = 7.98, p = 0.435; Table 2.4).

The specific mother (χ^2_1 = 0.03, p = 0.868; Table 2.5) and father (χ^2_1 = 2.11, p = 0.146) used in crosses did not significantly influence full-sib family sex ratios. The population of origin of mother plants was likewise not found to influence full-sib family sex ratios (χ^2_2 = 1.82, p = 0.403). However, a marginally significant difference was detected in sex ratios from crosses involving parents from different combinations of populations (χ^2_1 = 3.22, p = 0.073; Figure 2.3). Specifically, intra-population crosses between Spanish mothers and the Spanish father produced significantly more male-biased sex ratios than all other within- and between-population crosses. This would suggest that family sex ratios may vary in crosses involving parents from certain populations, but that population- and individual-level maternal and paternal effects do not display a consistent influence on sex ratios.

2.3.3 Inheritance of *OPB01-1562* and segregation with gender

Of the 15 parent plants used in controlled crosses, all three male plants, but none of the 12 females, amplified the DNA (SCAR) marker *OPB01-1562* (Figure 2.4). Of the 288 progeny tested (four male and four female progeny from each of the 36 crosses), all male individuals, but no females, amplified *OPB01-1562*. All individuals (parents and progeny), amplified the 766 bp control marker.

Table 2.1 Sex ratios (number of males/total number of individuals) of the 48 field-collected half-sib seed families, arranged in descending order of seed family size. See Appendix 9.1 for full details of the populations from which these families were sampled. * Denotes values of *G* significant at the table-wide $p < 0.05$ level (Dunn-Šidák method).

Population & seed family code	Country of origin	Males	Females	Total	Sex ratio	*G*
331.18	Spain	260	217	477	0.545	3.88
40a1	Spain	244	213	457	0.534	2.10
J11	Israel	156	193	349	0.447	3.93
584b2	Spain	134	142	276	0.486	0.23
331.1	Spain	99	98	197	0.503	0.01
BS10	UK	74	117	191	0.387	9.76
J14	Israel	86	95	181	0.475	0.45
BS11	UK	90	90	180	0.500	0.00
BS5	UK	84	70	154	0.545	1.27
J15	Israel	69	76	145	0.476	0.34
PS4	UK	74	65	139	0.532	0.58
PS3	UK	59	78	137	0.431	2.64
BS4	UK	62	51	113	0.549	1.07
31a2	Spain	41	57	98	0.418	2.62
BS1	UK	42	45	87	0.483	0.10
AR3	UK	53	30	83	0.639	6.46
40a4	Spain	44	31	75	0.587	2.26
331.15	Spain	37	37	74	0.500	0.00
AR6	UK	43	31	74	0.581	1.95
41a9	Spain	40	33	73	0.548	0.67
J10	Israel	39	33	72	0.542	0.50
TE17	Israel	28	36	64	0.438	1.00
TB35	Israel	33	29	62	0.532	0.26
34a1	Spain	42	16	58	0.724	12.08*
J13	Israel	17	34	51	0.333	5.78
330.18	Spain	22	29	51	0.431	0.96
330.27	Spain	26	25	51	0.510	0.02
331.13	Spain	24	25	49	0.490	0.02

Table 2.1 (continued)

Population & seed family code	Country of origin	Males	Females	Total	Sex ratio	*G*
TB63	Israel	27	21	48	0.563	0.75
584b1	Spain	21	23	44	0.477	0.09
41a15	Spain	22	16	38	0.579	0.95
40a2	Spain	19	18	37	0.514	0.03
TB22	Israel	16	20	36	0.444	0.45
RB6	Israel	22	14	36	0.611	1.79
19a12	Spain	12	22	34	0.353	2.99
37a6	Spain	14	19	33	0.424	0.76
RB7	Israel	11	22	33	0.333	3.74
41a16	Spain	14	16	30	0.467	0.13
31a8	Spain	14	15	29	0.483	0.03
31a3	Spain	17	12	29	0.586	0.87
331.11	Spain	13	15	28	0.464	0.14
RB11	Israel	12	11	23	0.522	0.04
31a7	Spain	6	10	16	0.375	1.01
37a5	Spain	10	6	16	0.625	1.01
34a10	Spain	9	6	15	0.600	0.60
CMa7	UK	4	8	12	0.333	1.36
41a7	Spain	5	6	11	0.455	0.09
CMa10	UK	5	5	10	0.500	0.00
Total		2295	2281	4576	0.502	
					$\mathbf{G_{total}}$	77.82
					$\mathbf{G_{het}}$	77.77
					$\mathbf{G_{pooled}}$	0.04

Table 2.2 Sex ratios (number of males/total number of individuals) of the three populations from which parent plants used in controlled crosses were sampled. See Appendix 9.1 for full details of population locations.

Population code	Population location	Males	Females	Total	Sex ratio	*G*
98a	HaGoshrim, Israel	111	108	219	0.507	0.04
232a	Sestri Levante, Italy	66	70	136	0.485	0.12
668b	Tarragona, Spain	123	135	258	0.477	0.56

Table 2.3 Sex ratios (number of males/total number of individuals) of the 36 full-sib seed families from controlled crosses. * Denotes values of *G* significant at the table-wide $p < 0.05$ level (Dunn-Šidák method).

Mother	Father	Males	Females	Total	Sex ratio	*G*
ItalyG1	ItalyM2	219	231	450	0.487	0.32
ItalyG2	ItalyM2	35	39	74	0.473	0.22
ItalyG3	ItalyM2	7	8	15	0.467	0.07
ItalyG4	ItalyM2	10	8	18	0.556	0.22
SpainG1	ItalyM2	4	6	10	0.400	0.40
SpainG2	ItalyM2	42	40	82	0.512	0.05
SpainG3	ItalyM2	5	14	19	0.263	4.44
SpainG4	ItalyM2	10	12	22	0.455	0.18
IsraelG1	ItalyM2	170	170	340	0.500	0.00
IsraelG2	ItalyM2	8	11	19	0.421	0.48
IsraelG3	ItalyM2	5	5	10	0.500	0.00
IsraelG4	ItalyM2	4	5	9	0.444	0.11
ItalyG1	SpainM2	6	6	12	0.500	0.00
ItalyG2	SpainM2	123	98	221	0.557	2.83
ItalyG3	SpainM2	64	54	118	0.542	0.85
ItalyG4	SpainM2	93	84	177	0.525	0.46
SpainG1	SpainM2	256	211	467	0.548	4.34
SpainG2	SpainM2	138	111	249	0.554	2.93
SpainG3	SpainM2	61	46	107	0.570	2.11
SpainG4	SpainM2	77	52	129	0.597	4.88
IsraelG1	SpainM2	10	14	24	0.417	0.67
IsraelG2	SpainM2	233	254	487	0.478	0.91
IsraelG3	SpainM2	244	239	483	0.505	0.05
IsraelG4	SpainM2	60	52	112	0.536	0.57
ItalyG1	IsraelM2	203	160	363	0.559	5.11
ItalyG2	IsraelM2	344	330	674	0.510	0.29
ItalyG3	IsraelM2	140	150	290	0.483	0.34
ItalyG4	IsraelM2	167	155	322	0.519	0.45
SpainG1	IsraelM2	206	173	379	0.544	2.88
SpainG2	IsraelM2	117	194	311	0.376	19.26*

Table 2.3 (continued)

Mother	Father	Males	Females	Total	Sex ratio	*G*
SpainG3	IsraelM2	173	191	364	0.475	0.89
SpainG4	IsraelM2	214	232	446	0.480	0.73
IsraelG1	IsraelM2	316	347	663	0.477	1.45
IsraelG2	IsraelM2	341	320	661	0.516	0.67
IsraelG3	IsraelM2	275	271	546	0.504	0.03
IsraelG4	IsraelM2	135	113	248	0.544	1.95
Total		4515	4406	8921	0.506	
					$\mathbf{G_{total}}$	61.13
					$\mathbf{G_{het}}$	59.80
					$\mathbf{G_{pooled}}$	1.33

Table 2.4 Sex ratios (number of males/total number of individuals) of the eight further crosses undertaken using progeny from the significantly biased full-sib seed family SpainG2 x IsraelM2.

Mother	Father	Males	Females	Total	Sex ratio	*G*
Female 2	Male 5	68	50	118	0.576	2.76
Female 6	Male 5	19	12	31	0.613	1.59
Female 8	Male 5	40	48	88	0.455	0.73
Female 14	Male 5	150	152	302	0.497	0.01
Female 2	Male 11	19	13	32	0.594	1.13
Female 6	Male 11	24	31	55	0.436	0.89
Female 8	Male 11	148	153	301	0.492	0.08
Female 14	Male 11	37	45	82	0.451	0.78
Total		505	504	1009	0.500	
					$\mathbf{G_{total}}$	7.98
					$\mathbf{G_{het}}$	7.98
					$\mathbf{G_{pooled}}$	0.00

Table 2.5 Results of the generalized linear mixed effects model (GLMM) analysing the sex ratios of full-sib seed families from controlled crosses. Values for main effects calculated following the removal of the marginally significant MotherPop x Father interaction term.

	d.f.	χ^2	***p***
MotherPop	2	1.82	0.403
Father	1	2.11	0.146
Mother(MotherPop)	1	0.03	0.868
MotherPop x Father	1	3.22	0.073

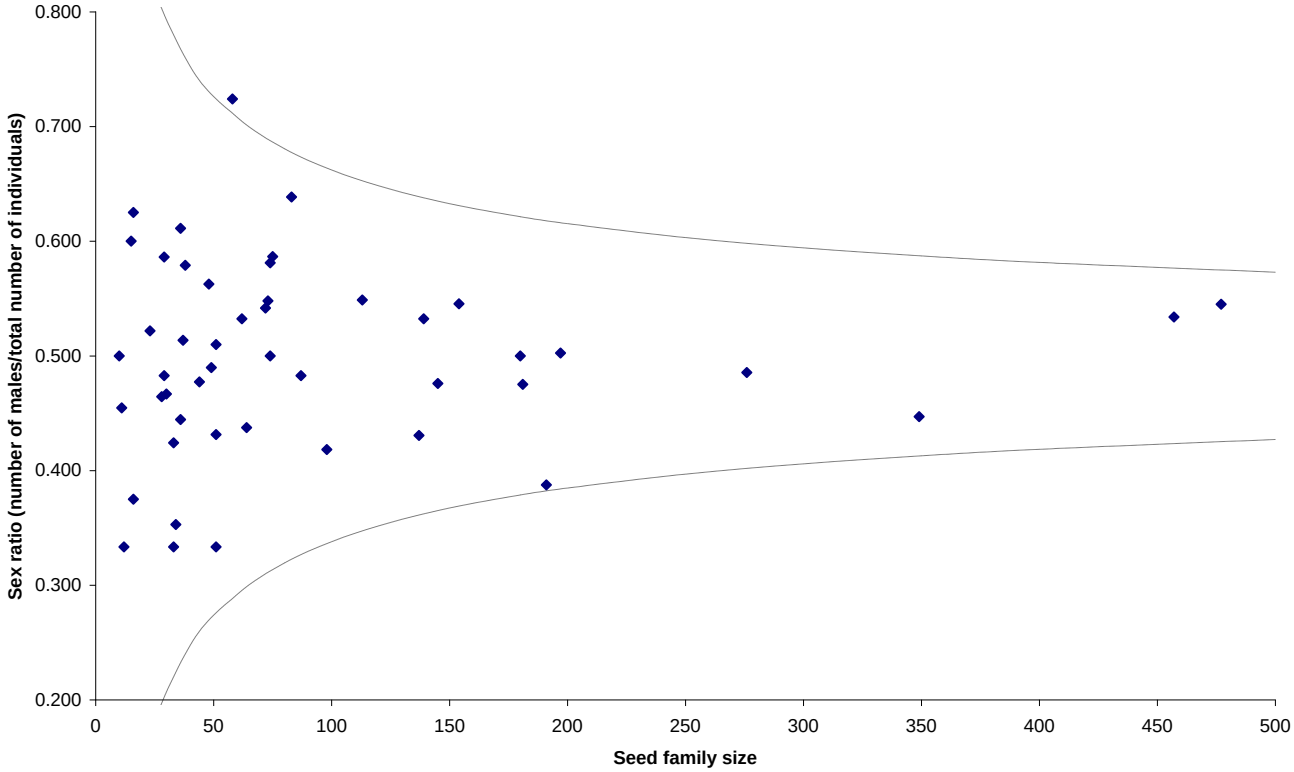


Figure 2.1 The sex ratios of the 48 field-collected half-sib seed families plotted against seed family size. Dashed lines represent the boundaries of the 0.05 acceptance region for tests of individual seed family sex ratios for departures from 1:1 (Dunn-Šidák method).

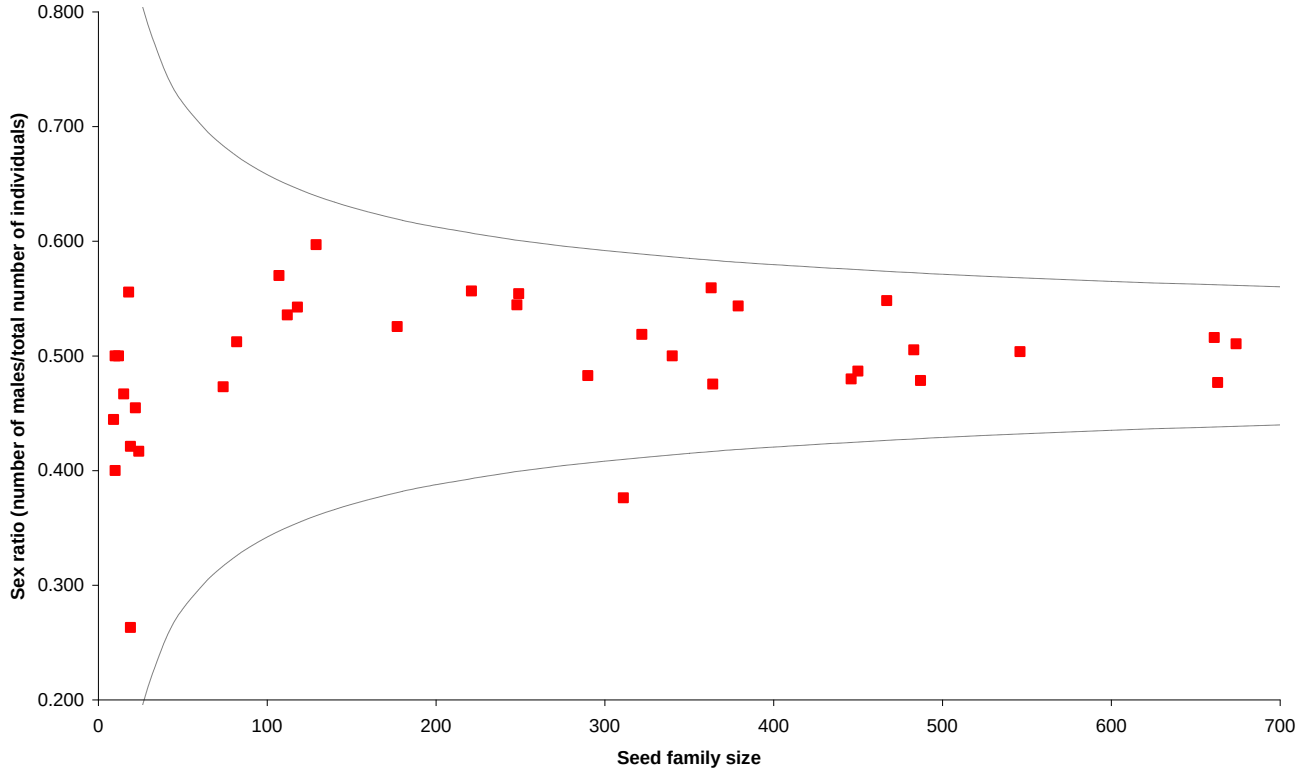


Figure 2.2 The sex ratios of the 36 full-sib seed families from controlled crosses plotted against seed family size. Dashed lines represent the boundaries of the 0.05 acceptance region for tests of individual seed family sex ratios for departures from 1:1 (Dunn-Šidák method).

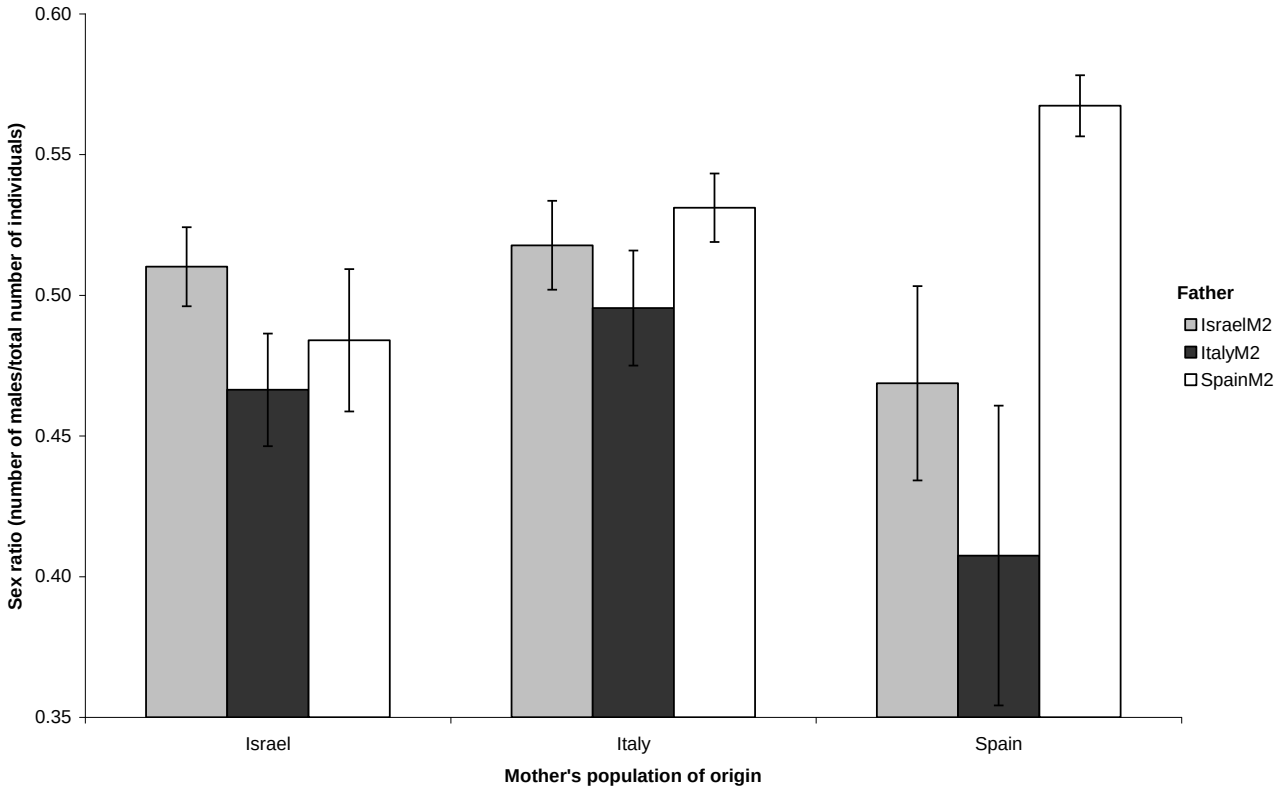


Figure 2.3 The mean sex ratios (± one standard error) of the nine within- and between- population crosses.

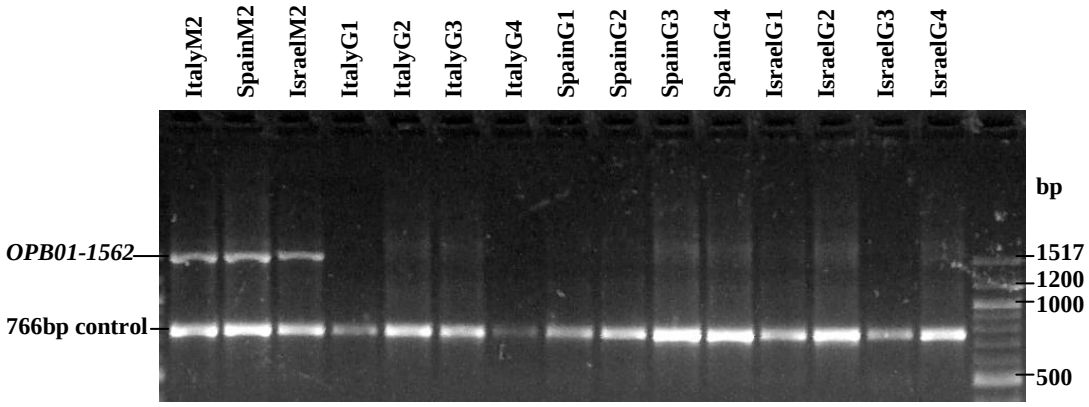


Figure 2.4 PCR amplification of *OPB01-1562* and a 766 bp control marker in the 15 parent plants used in controlled crosses. Right hand lane contains fragment size calibration ladder (100bp DNA Ladder, New England Biolabs).

2.4 DISCUSSION

2.4.1 Single-locus sex determination in dioecious *M. annua*

Overall, my results suggest that sex in dioecious *M. annua* is determined by a single-locus genetic mechanism. The results from analyses of both half-sib and full-sib seed family sex ratios are, to some extent, ambiguous in terms of distinguishing between single- and multi-locus mechanisms of sex determination; the lack of any deviation from 1:1 in the overall sex ratios of all half-sib and full-sib seed families points towards a single-locus system, yet the significant heterogeneity observed among all sex ratios indicates that a more complicated mechanism may be in operation. Three of the 84 individual seed families studied (two half-sib and one full-sib) had sex ratios significantly different from 1:1, although no deviation from 1:1 was observed in any of the seed families produced from crosses between the progeny of the biased full-sib seed family, suggesting there to be no simple heritable basis for this skew. Individual plants (both mothers and fathers) were also found not to significantly influence full-sib family sex ratios from controlled crosses, a finding consistent with a single-locus mechanism, whereas under a multi-locus system one may predict certain plants to yield consistently biased sex ratios according to the specific sex-determining alleles they possess. In contrast with these inconsistencies, however, the results from the analysis of the inheritance and segregation with gender of *OPB01-1562* point unequivocally toward a single-locus mechanism of sex determination in the species.

OPB01-1562 was found to co-segregate exclusively with male sex in all parents and progeny tested from each of the 36 controlled crosses. This result very strongly indicates a single-locus mechanism of sex determination to be operating in the

species; *OPB01-1562* is putatively linked to a dominant male-determining allele, with males being the heterogametic sex and females being homozygous recessive at this locus. That *OPB01-1562* was amplified in male individuals from all three populations sampled suggests there is no geographic variation in the species' sex-determining mechanism, a conclusion also supported by the fact that half-sib family sex ratios did not vary significantly between Israel, Spain and the United Kingdom. (The differences observed in full-sib family sex ratios from crosses involving parents from different combinations of populations can be explained by alternative factors; see below.) I therefore conclude that sex determination in dioecious *M. annua* is controlled by a single-, rather than multi-, locus genetic mechanism.

M. annua thus appears to conform to theoretical predictions for the evolution of single-factor sex determination following the evolution of dioecy from hermaphroditism. It is also therefore interesting to consider the extent of recombination suppression around the sex-determining locus in the species, which may be predicted to develop following the evolution of single-locus sex determination in order to prevent the break up of gene combinations conferring complementary sterility and functionality in male and female reproductive traits (see section 2.1; Rice, 1987a; 1996; Charlesworth, 2002). The lack of observable heterochromosomes (Durand, 1963) would indicate that recombination is not suppressed over a large chromosomal region in dioecious *M. annua*. However, as *OPB01-1562* was exclusively male-linked in all individuals tested during the course of this investigation and by Khadka *et al.* (2002; 2005), with no recombinants of *OPB01-1562* and sex having ever been detected, one may suppose that either: i) the marker is extremely tightly linked to the male-determining locus; ii) the marker is

itself a sex-determining gene (although this is unlikely given the reported homology of *OPB01-1562* to retrotransposons; see section 3.4; Khadka *et al.*, 2005); or iii) recombination is suppressed to some degree around the sex-determining locus, with *OPB01-1562* located within the non-recombining region. In the event that a degree of recombination suppression does exist, then the sex-determining chromosomes of dioecious *M. annua* may be at a similar evolutionary stage to those of *A. officinalis* (Telgmann-Rauber *et al.*, 2007) or *C. papaya* (Liu *et al.*, 2004), in which recombination is suppressed only across a small region around the sex-determining loci, with chromosomes still appearing homomorphic at the cytological level. Nonetheless, genetic mapping of the sex-determining region and the identification of further sex-linked markers in dioecious *M. annua* is necessary before any firm conclusions about the extent of recombination suppression can be made. If recombination suppression is restricted to a relatively small region around the sex-determining locus of dioecious *M. annua* (or even absent), then this raises the interesting question of why it has not evolved or expanded and why heteromorphic sex chromosomes have not developed in this species, in common with so many other dioecious plants? This question is discussed further in the following chapter of this thesis, in which I also undertake a search for new sex-linked DNA markers in the species.

2.4.2 Family sex ratio variation in dioecious *M. annua*

The use of a genetic marker provides strong evidence of single-locus sex determination, emphasising the advantages of molecular analyses over classic analyses of sex ratios as a tool for studying sex determination. Nonetheless, analyses of family sex ratios were informative in identifying departures from 1:1 that

point to the role of additional factors influencing the sex ratios of at least some families. Similar, indeed more extreme, examples of such deviations from 1:1 sex ratios are well documented in other dioecious plant species and can operate in spite of strict genetic sex-determining systems (reviewed in Werren & Beukeboom, 1998; de Jong & Klinkhamer, 2002; Barrett et *al*., 2010). For example, *Rumex* sp. (Rychlewski & Zarzycki, 1975; Stehlik & Barrett, 2005; Stehlik *et al.*, 2008), *S. latifolia* (Taylor, 1994a) and *Urtica dioica* (de Jong & Klinkhamer, 2002) all display family-level sex ratio heterogeneity despite the presence of heteromorphic sex chromosomes or, in the case of dioecious *U. dioica* (Glawe & de Jong, 2008), single-locus sex-determination. The heterogeneity detected amongst half-sib and full-sib seed family sex ratios in this study, plus the variation detected in full-sib family sex ratios from crosses involving parents from different combinations of populations, are likely to be caused by similar factors.

Two major mechanisms have been proposed to account for biases and heterogeneity in seed family sex ratios, namely: 1) certation, the differential performance of male- and female-determining pollen grains due to the accumulation of mutations in degenerate Y chromosomes (Correns, 1922); and 2) meiotic drive/segregation distortion, a form of intragenomic conflict leading to a bias in the ratio of male- to female-determining gametes produced (Taylor & Ingvarsson, 2003). For certation to occur, a degree of chromosome degeneration is believed to be required to produce differences in the performance of X- versus Y-bearing microgametophytes. As *M. annua* lacks heteromorphic sex chromosomes (Durand, 1963), it is unlikely to be a process operating in this species. Furthermore, certation is thought to be capable only of producing female-biased, not male-biased, sex ratios in species with XY sex

determination, as it is the male (Y)-determining pollen which is of lower fitness relative to female (X)-determining pollen. In this study, both male- and female-biased family sex ratios were observed, with no departure from 1:1 in the overall sex ratio, thus suggesting that certation is probably not occurring in dioecious *M. annua*.

Alternatively, the deviations from 1:1 sex ratios observed here could be attributed to male- or female-linked sex ratio distorters, 'selfish' genetic elements which modify the sex ratio to promote their own transmission. This phenomenon has been well studied in *S. latifolia*, where family sex ratios are often female-biased. Through the use of reciprocal crosses, Taylor (1994a) indicated that the extent of the female-bias was influenced largely by a Y-linked sex ratio modifier that increases the proportion of males in the progeny to counteract the effects of X-linked and cytoplasmic feminizing genes. A similar mechanism may be operating in *M. annua*, as shown by the differences detected in full-sib family sex ratios from crosses involving parents from different combinations of populations. Given that dioecious *M. annua* is believed to have undergone postglacial range expansion and displays a geographic gradient in genetic diversity (Obbard *et al*., 2006c), variation in sex ratio distorters might exist between geographically distant populations. When individuals from distant populations are crossed, one might therefore expect highly heterogeneous sex ratios as different distorter and modifier genes are brought together. This idea is supported by the fact that population- and individual-level maternal and paternal effects did not display a consistent influence on full-sib family sex ratios, suggesting that effects on sex ratios vary in crosses between parents from certain populations. For example, only when certain combinations of distorter and modifier genes from divergent populations are brought together may biased sex ratios be produced.

Nevertheless, it was an intra-population cross (between Spanish mothers and the Spanish father) that produced a significantly different sex ratio compared to all other within- and between-population crosses, although it is possible that unique distorters are present in this population which act only in synergy to produce biased sex ratios. Ideally, more fathers from each population should be tested in order to differentiate between population- and individual-level paternal effects on sex ratios.

The inheritance of sex ratio distorters at multiple nuclear or cytoplasmic loci, interacting to distort sex ratios away from 1:1, may also explain the significant heterogeneity observed in half-sib and full-sib family sex ratios, as well as the occurrence of three significantly biased individual seed families. It is worth noting, however, that the eight further crosses undertaken between F_1 progeny of the biased full-sib seed family produced no biased sex ratios in the F_2 generation, thus suggesting no heritable basis for the female skew. Of course, it remains possible that at least some of the significant deviations from 1:1 were the result of Type I error. Reciprocal crosses, similar to those used by Taylor (1994a) in *Silene*, would thus be informative in formally investigating whether sex ratio distorters are present in dioecious *M. annua*. Nevertheless, I conclude that gender in dioecious *M. annua* is controlled by a single-locus genetic mechanism, with additional factors potentially influencing sex ratios at the individual family level.

2.4.3 Comparisons with the multi-locus model

Despite rejecting the three-locus model, I believe the work of Louis (1989) does, to a certain extent, correlate with my results. The majority of male x female crosses performed by Louis (1989) yielded 1:1 sex ratios, while most male x male crosses

produced 3:1 sex ratios, as would be predicted under single-locus sex determination if putative 'YY' genotypes are viable, as they often are in species lacking heteromorphic sex chromosomes (Westergaard, 1958; Charlesworth, 2002). The three-locus model was proposed largely as a result of departures from 1:1 and 3:1 sex ratios, but no account was taken of the high number of crosses (> 60) performed in Louis' study, amongst which some deviation would be expected purely by chance. Furthermore, if sex ratio distorters are indeed present in dioecious *M. annua*, then this could have further contributed to the reported biases. As Louis self-fertilised and inbred several *M. annua* male lines, abnormal sex ratios may have been produced if distorter genes were present and inherited in combinations which do not arise in wild populations.

Moreover, with regard to the role of the *A*, *B1* and *B2* loci proposed by Louis (1989), it is well established that phytohormones play a key role in sex determination in *M. annua* (Louis, 1989; Durand & Durand, 1991). In particular, auxins and cytokinins have been identified as influencing gender development in the species, two groups of hormones which act antagonistically and have long been known to regulate plant growth, development and sexual expression (Skoog & Miller, 1957; Yamasaki *et al.*, 2005). The three-locus mechanism was based on differences in 'male strength' (the degree of resistance to feminization by the exogenous application of cytokinins) between male *M. annua* individuals. The inheritance of different combinations of alleles at the two *B* loci was believed to explain these differences: *B1* associated with *B2* induces complete resistance to feminization; *B1* alone confers intermediate resistance; whilst *B2* alone gives low resistance (Louis, 1989). Furthermore, endogenous auxin and cytokinin levels have been reported to be correlated with

different allelic combinations of the three sex genes (Hamdi *et al.*, 1987; Louis *et al.*, 1990). The single sex-determining locus identified in this study might therefore represent the *A* gene of the multi-locus model; inheritance of a dominant allele at this locus is necessary for the development of males in both models. It is possible, then, that the *B* genes in the three-locus model are regulators of auxin production, which are activated by, and act downstream of, the *A* gene. There are potentially numerous such *B* genes throughout the *M. annua* genome, though particular alleles might be locally fixed in some populations, so that they would not display the polymorphism of those identified by Louis (1989). Inheritance of different allelic combinations of *B1* and *B2* would thus influence endogenous auxin and cytokinin levels, but not affect the gender of the plant. The adaptive reasons for differences in the levels of these phytohormones between individuals are unclear, however. In short, I speculate that Louis' *A* gene represents the single sex-determining locus in *M. annua*, to which *OPB01-1562* is putatively linked, and that *B1* and *B2* may be two of a (potentially large) class of genes that control sexual expression through phytohormone regulation, but do not ultimately control sex determination.

2.4.4 Conclusions

The evidence presented above indicates that sex determination in dioecious *M. annua* is controlled by a single-locus genetic mechanism, with males the heterogametic sex and the DNA marker *OPB01-1562* putatively linked to a dominant male-determining allele. This finding raises a number of interesting questions. To what extent, for example, is recombination suppressed around the putative sex-determining locus, as might be predicted to occur following the establishment of a single-locus system of sex determination? If recombination is not

suppressed, or only suppressed across a very small region, why is this the case, and why does dioecious *M. annua* not possess heteromorphic sex chromosomes like *S. latifolia*? Moreover, with specific regard to annual mercuries, has this single-locus mechanism of sex determination been conserved across the clade (in which dioecy is believed to be the ancestral state; Durand & Durand, 1992; Krähenbühl *et al.*, 2002; Obbard *et al*., 2006b), in polyploid lineages of the *M. annua* species complex (particularly the androdioecious system), or even across the *Mercurialis* genus? Polyploid lineages within the *M. annua* species complex show great diversity in sexual system and are believed to have undergone hybridisations and polyploidisations (Obbard et *al*., 2006b); how has this mechanism of sex determination been affected by such changes? Furthermore, hexaploid androdioecious *M. annua* has been reported to possess a mixed genetic-environmental system of sex determination (Pannell, 1997a); how may the single-locus genetic mechanism identified in this study have been modified to enable individuals to also respond to growing density? I address these questions in the following chapters of this thesis.

Another question to be raised by this study is whether multi-locus systems of sex determination are able to evolve and be maintained in dioecious plant species? With the identification of single-locus sex determination in *M. annua*, I am unaware of any dioecious species reported to possess a multi-locus system of sex determination. Under the constraints imposed during the evolution of unisexuality, multi-locus and polygenic mechanisms of sex determination are thought to be difficult to evolve (see section 2.1). Selection for tight linkage between male- and female-determining genotypes will promote the development of single-locus, or 'monogenic', sex

determination, as found in a variety of dioecious species. Study of the evolution and maintenance of polygenic sex determination in the few animal species in which it has been reported would be a logical starting point for investigation of this question. Indeed, recent modelling work by Blaser *et al.* (2011) has suggested that strong sexual selection may permit maintenance of poly-factorial sex determination in fish, though empirical data is lacking. Further studies in this area may thus improve our understanding of the evolution of sex-determining mechanisms across the tree of life.

3. SEX-LINKED DNA MARKERS IN THE ANNUAL MERCURIES

3.1 INTRODUCTION

Recent advances in the ability to develop genetic markers in non-model species have enabled sex-linked DNA markers to be identified in a range of taxa (e.g. Peichel *et al.*, 2004; Spigler *et al.*, 2008; Smith & Voss, 2009; Charlesworth & Mank, 2010). Such markers are particularly important for addressing questions relating to the evolution of sex chromosomes (Charlesworth *et al*., 2005; Fraser & Heitman, 2005), the consequences of recombination suppression (Charlesworth, 1996; Bergero & Charlesworth, 2009), the adaptation of sex-linked genes (Rice, 1984; Charlesworth *et al.*, 1987; Vicoso & Charlesworth, 2006; Mank, 2009), and the conservation of mechanisms of sex determination and specific sex-determining genes amongst related taxa (Ferguson-Smith, 2007; Mrackova *et al.*, 2008; Williams & Carroll, 2009). In plants, the sex-determining regions of very few species have been genetically mapped to date. *Silene latifolia* is a notable exception, a dioecious species with heteromorphic sex chromosomes in which up to 1,800 putatively sex-linked genes have recently been identified (Bergero & Charlesworth, 2011; Chibalina & Filatov, 2011; Muyle *et al.*, 2012). The identification of numerous sex-linked markers, and, specifically, active sex-linked genes in *S. latifolia* has permitted detailed study of sex chromosome evolution in the species, including of: Y chromosome genetic degeneration and the conservation of gene function (Marais *et al.*, 2008; Bergero & Charlesworth, 2011; Chibalina & Filatov, 2011); nucleotide diversity in sex-linked versus autosomal loci (Filatov *et al.*, 2001; Qiu *et al.*, 2010);

gene divergence between X- and Y-linked homologues (Filatov *et al.*, 2000; 2001; Laporte *et al.*, 2005; Kaiser *et al.*, 2011); dosage compensation (mechanisms which equalise expression of X-linked genes in males and females; Chibalina & Filatov, 2011; Muyle *et al.*, 2012); evolutionary strata (distinct regions of the non-recombining region which together show a stepwise decrease in divergence time between X- and Y-linked homologues; Bergero *et al.*, 2007; Chibalina & Filatov, 2011); population genetic structure and differentiation of sex chromosomes (Ironside & Filatov, 2005; Muir *et al.*, 2011); and the evolution of sex-determining mechanisms and sex chromosomes across the genus (Mrackova *et al.*, 2008; Marais *et al.*, 2011). *S. latifolia* represents something of an exception, however, since in general, sex-linked markers have proved more challenging to find in plants than in other taxa owing to the small sex-specific genomic regions which typify many dioecious species.

In many plant species, recombination suppression around sex-determining loci has either not evolved or is restricted to a small region, with little or no chromosome degeneration (Charlesworth *et al.*, 2005; Charlesworth & Mank, 2010; Ming *et al.*, 2011), e.g. in *Asparagus officinalis* (Telgmann-Rauber *et al.*, 2007), *Spinacia oleracea* (Khattak *et al.*, 2006; Lan *et al.*, 2006) and *Fragaria virginiana* (Spigler *et al.*, 2008). Reasons for this remain unclear (Charlesworth, 2002; 2008), although it has been postulated that the recent origin of dioecy in some species, or a low degree of sexual antagonism in plants, which may reduce the accumulation of sexually antagonistic mutations around the sex-determining region, are responsible for the rarity of heteromorphic sex chromosomes among dioecious species (see section 2.1; Charlesworth, 2008; Charlesworth & Mank, 2010). Alternatively, the unique

lifecycle of plants (which involves alteration between diploid and haploid generations) has also been proposed to prevent sex chromosome divergence and degeneration, with purifying selection acting on genes expressed during the gametophytic (haploid) stage preserving gene integrity (Charlesworth, 2008; Chibalina & Filatov, 2011). Whatever the factors preventing the expansion of non-recombining regions around sex-determining genes in plants, few, if any, fully sex-linked markers have been identified in many dioecious species as a result, even with fine-scale mapping (Charlesworth & Mank, 2010).

The recent identification of a fully sex-linked DNA marker in dioecious *Mercurialis annua* (*OPB01-1562*; Chapter 2; Khadka *et al.*, 2002) presents an opportunity to study the evolution of sex determination in both *M. annua* and in annual mercuries more widely. For example, given that dioecy is believed to be the ancestral state in the annual mercury clade, and possibly in the genus *Mercurialis* (molecular evidence indicates dioecious species to be basal in the *Mercurialis* phylogeny, plus in the *M. annua* species complex dioecious populations are diploid whereas monoecious and androdioecious populations are polyploid; Durand & Durand, 1992; Krähenbühl *et al.*, 2002; Obbard *et al.*, 2006b), it is interesting to consider whether the sex-linkage of *OPB01-1562* has been conserved amongst dioecious annual mercuries, or even across the genus as a whole. Evidence of the sex-linkage of *OPB01-1562* in other dioecious *Mercurialis* species would suggest there to be a conserved mechanism of single-locus sex determination across the genus, with *OPB01-1562* putatively linked to a male-determining allele ubiquitous amongst dioecious *Mercurialis* species. Conversely, the absence of *OPB01-1562* in other *Mercurialis* species might simply reflect genomic divergence between species at this locus, but could alternatively

point towards modification of the sex-determining mechanism between species, or even suggest multiple independent origins of sex-determining mechanisms in the genus, as has been reported in *Silene* (Mrackova *et al*., 2008; Marais *et al*., 2011) and *Rumex* (Navajas-Pérez *et al*., 2005).

In addition to study of this male-linked marker in dioecious species of *Mercurialis*, it is also interesting to examine whether *OPB01-1562* has been conserved in polyploid lineages of *M. annua*, given the diversity in mating systems they present and the hypothesised evolutionary history of the annual mercury clade (see section 1.2; Obbard *et al*., 2006b). Identification of *OPB01-1562* in polyploid lineages may shed light on how the sex-determining mechanism of diploid *M. annua* (the hypothesised progenitor of autotetraploid, and subsequently allohexaploid, *M. annua*; Figure 1.2; Obbard *et al*., 2006b) has been modified to give rise to monoecious and androdioecious sexual systems. This question is particularly interesting with regard to androdioecious populations, given the occurrence of males. It is supposed that, as the hypothesised paternal progenitor of allohexaploid *M. annua*, the male-determining genotype of androdioecious *M. annua* was inherited from *M. huetii* (Obbard *et al*., 2006b). As theory predicts androdioecy should be difficult to evolve and maintain directly from hermaphroditism, owing to the need for males to sire at least twice the number of progeny of hermaphrodites (Lloyd, 1975; Charlesworth & Charlesworth, 1978a; Charlesworth, 1984; Pannell, 2002), evidence of the inheritance of a fully functional male-determining genotype from *M. huetii* may account for the origin of this rare mating system. Assessing individuals from androdioecious populations of *M. annua* for the presence of a sex-linked marker potentially conserved across the annual mercury clade may thus help elucidate the

origin of androdioecy in the species. Khadka *et al*. (2002) tested individuals from monoecious and androdioecious populations of hexaploid *M. annua* (including male hexaploids) for the presence of *OPB01-1562* by polymerase chain reaction (PCR) amplification but found the marker did not amplify in any hexaploid plants. I here, however, test for the presence of *OPB01-1562* amongst a wider sample of annual mercury lineages, including the dioecious species *M. huetii* (the sister species of *M. annua*) and *M. canariensis*, using both PCR amplification and DNA blot hybridisation analysis in order to fully assess the conservation of this marker across the annual mercury clade.

In addition to this study, I also undertake a search to identify new sex-linked genes in dioecious *M. annua* through the use of a segregation analysis of sequenced gene transcripts from two crosses of dioecious *M. annua*. This approach was used to identify up to 1,800 putatively sex-linked genes in *S. latifolia* (Bergero & Charlesworth, 2011; Chibalina & Filatov, 2011; Muyle *et al.*, 2012) and thus has the potential to identify numerous sex-linked genes, some of which may be conserved across the annual mercury clade, or even across the *Mercurialis* genus.

3.2 MATERIALS AND METHODS

3.2.1 Assessing the conservation of *OPB01-1562* by PCR amplification

Seeds of *M. huetii*, *M. canariensis* and diploid, tetraploid and hexaploid (androdioecious) *M. annua* for this experiment were bulk collected from a single population of known ploidy for each lineage (ploidy previously assessed by Obbard *et al*., 2006b; see Appendix 9.1 for full details of populations used). Approximately 50 seeds from each population were sown in soil-based compost in separate seed trays and grown under standard glasshouse conditions. Following germination and flowering, genomic DNA was extracted from fresh leaf material using a modified cetyltrimethylammonium bromide (CTAB) procedure (specific modifications are described in section 2.2.4; Doyle & Doyle, 1987). DNA was extracted from eight individuals of each gender per lineage. All individuals were assessed for the presence of the DNA (SCAR) marker *OPB01-1562* and for a 766 bp control marker (identified by Khadka *et al.*; 2002) by PCR amplification with primer pairs B1F01/B1R01 and B1F01/B1R06 respectively (Khadka *et al.*, 2002). PCR reagents and amplification conditions were identical to those described in section 2.2.4 of this thesis. PCR products were visualized by ethidium bromide staining after electrophoresis on 1.5% agarose gels.

3.2.2 Assessing the conservation of *OPB01-1562* by DNA blot hybridisation analysis

Genomic DNA for this experiment was extracted from fresh leaf material of plants from each of the populations grown in section 3.2.1. Specifically, DNA was extracted from the pooled leaves of eight individuals of each gender per population using a modified urea-based procedure (Chen & Dellaporta, 1994), with phenol-

chloroform (1:1 by volume) used for organic solvent extraction. 7 µg DNA of each gender per lineage was then digested with each of the following restriction endonucleases: *Hin*dIII (Promega); *Xba*I; *Kpn*I; and *Pst*I (all New England Biolabs). Digests were electrophoresised at 50 V on 0.8% agarose gels overnight and denatured DNA was transferred to Nytran Nylon N membranes (Whatman) by Southern blot.

OPB01-1562 was prepared for use as a probe in DNA blot hybridisations by cloning the purified product obtained from PCR of diploid male *M. annua* genomic DNA with primer pair B1F01/B1R01 (described in section 3.2.1) in the pGEM-T Easy Vector (Promega). The probe was excised from the vector by *Eco*RI digestion, radio-labelled with [α–^{32}P]dCTP using the Rediprime II DNA Labeling System (GE Healthcare) and hybridised with the DNA blots overnight at high stringency (65°C). Hybridised blots of diploid *M. annua* DNA were then washed at high stringency (first and second washes 1X SSC, 0.2% SDS, 65 ºC, 45 min), whilst all other blots were washed at low stringency (first wash 2X SSC, 0.2% SDS, 58 ºC, 45 min; second wash 1X SSC, 0.2% SDS, 58 ºC, 45 min). Hybridisation of the radio-labelled *OPB01-1562* probe to genomic DNA fragments was detected by exposing blots to X-ray film for 48 hours and developing films by hand.

3.2.3 Identifying new sex-linked genes in dioecious *M. annua*

Seeds for crosses for this study were bulk collected from > 300 female individuals of a single population of dioecious *M. annua* located in Castelldefels sud, Spain (Appendix 9.1). Approximately 50 seeds from this population were sown in a single seed tray of soil-based compost and, following germination and flowering, one male

and two female plants were selected at random and transplanted into individual 15 cm diameter pots. Once transplants were established, individuals were crossed by placing the three pots inside a pollen-proof growth box, in which plants were watered and maintained under standard glasshouse conditions for six weeks to permit open pollination of the female plants by the male. After six weeks' growth, seeds from the two female plants were collected and subsequently grown in separate seed trays of soil-based compost.

To identify new sex-linked genes in dioecious *M. annua*, the poly-A transcriptomes of the three parents, plus five male and five female progeny selected at random from each cross, were sequenced. Total RNA was extracted from shoots and flower buds of all individuals using an RNeasy Plant Mini Kit (Qiagen) with on-column DNase digestion. Isolation of polyA-RNA, cDNA synthesis and high-throughput sequencing were conducted following standard *Illumina* RNA-Seq procedure, using an *Illumina* GAII instrument for sequencing with 100 base paired end reads. All parents and progeny were sequenced using a single *Illumina* lane per individual, with a coverage per sample of at least 20 million reads. A reference sequence for the *M. annua* poly-A transcriptome was assembled using a single sample with good coverage (a read count of 43,082,244 paired end reads) in *CLC Genomics Workbench 4* (CLC bio). A minimum contig length of 500 bases yielded 20,412 contigs (N50 = 1821; N90 = 792), against which reads from all other transcriptomes sequenced could be mapped.

Sex-linked genes were identified by segregation analysis of single nucleotide polymorphisms (SNPs) in the two crosses. SNPs were identified in parents and their

segregation with gender in the progeny of crosses was traced using the program *Segregator* (Chibalina & Filatov, 2011). For SNP detection, a position was required to be covered by at least five sequence reads in each individual to avoid SNP-calling errors, though coverage for most SNPs was much greater. Sex-linked genes were inferred from SNP segregation patterns as follows: SNPs which were inherited exclusively from father to daughters (and not sons) were deemed to be in X-linked genes; SNPs which were inherited exclusively from father to sons (and not daughters) were deemed to be Y-linked (for further details see Chibalina & Filatov; 2011).

To confirm the sex-linkage of genes identified by this method, one putatively X-linked and five putatively Y-linked genes were randomly selected and sex-linkage in parents and progeny from crosses tested by manual low-throughput approach. Genomic DNA was extracted from fresh and frozen leaf material of all three parents and five male and five female progeny per cross using a DNeasy Plant Mini Kit (Qiagen). Primers for putatively sex-linked genes were designed using the program *Primer3* (Rozen & Skaletsky, 2000) and regions of the six genes were amplified in all individuals by PCR. Full details of genes tested and primer sequences are shown in Table 3.1. PCR reagents and amplification conditions were identical to those described in section 2.2.4. PCR products were then sequenced in all individuals using BigDye terminator version 3.1 (Applied Biosystems), following the manufacturer's instructions, and the presence and segregation with gender of SNPs was confirmed visually using *Sequencher* version 4.5 (Gene Codes).

Following confirmation of sex-linkage of the six genes initially tested in parents and progeny from crosses, ten putatively Y-linked genes were tested for sex-linkage in dioecious *M. annua* individuals sampled from wild populations across the species' geographic range. Of the ten genes tested, five were selected based on a divergence of > 1.3% from their X-linked homologues in order to increase the likelihood of identifying a fully male-linked gene in the species; genes more closely linked to the sex-determining region are predicted to be more divergent from X-linked homologues as a result of experiencing longer periods of recombination suppression relative to genes located further along the sex-determining chromosome pair, assuming recombination suppression does exist in *M. annua* and a gradual or stepwise expansion of the non-recombining region (Lahn & Page, 1999; Bergero & Charlesworth, 2009). Specifically, seeds were bulk collected from six populations of dioecious *M. annua* (see Appendix 9.1 for full details of populations used) and grown in seed trays of soil-based compost. Genomic DNA was extracted from two male and two female individuals per population using the modified CTAB procedure described in section 2.2.4 (Doyle & Doyle, 1987) and regions of each of the ten putatively Y-linked genes were amplified in all individuals by PCR using a mixture of Y-specific primers and primers conserved between X and Y gene homologues designed using *Primer3* (Table 3.1; Rozen & Skaletsky, 2000; PCR reagents and amplification conditions as described in section 2.2.4). PCR products were visualized by ethidium bromide staining after electrophoresis on 1.5% agarose gels and were sequenced in all individuals using BigDye terminator version 3.1 (Applied Biosystems), with the presence of SNPs assessed visually in sequences using *Sequencher* version 4.5 (Gene Codes).

3.3 RESULTS

3.3.1 PCR amplification of *OPB01-1562*

All eight male diploid *M. annua* individuals, but none of the eight females, successfully amplified *OPB01-1562*. In contrast, no *M. huetii*, *M. canariensis*, tetraploid or hexaploid *M. annua* individuals amplified *OPB01-1562*, irrespective of the gender of individuals (Figure 3.1). All individuals tested amplified the 766 bp control marker.

3.3.2 DNA blot hybridisation analysis

DNA blots of genomic DNA from *M. huetii*, *M. canariensis* and diploid, tetraploid and hexaploid *M. annua* individuals of separate sexes were successfully hybridised with *OPB01-1562* (Figure 3.2). All hybridising bands were conserved between sexes in each lineage, with no sex-specific polymorphisms detected, including in diploid *M. annua*. The sequence polymorphism(s) that allowed sex-specific PCR amplification in diploid *M. annua* were thus not detected using any of the restriction enzymes used here. Blots in which DNA was digested using *Hin*dIII, *Xba*I or *Kpn*I restriction enzymes produced multiple bands of strong signal (≥ 3 in each case), potentially indicating the presence of multiple copies of regions of *OPB01-1562* in the genomes of the lineages tested.

3.3.3 New sex-linked genes in dioecious *M. annua*

464 putatively sex-linked genes were identified in dioecious *M. annua* by segregation analysis of sequenced gene transcripts. The sex-linkage of one putatively X-linked and five putatively Y-linked genes were successfully confirmed in parents and progeny from crosses by manual low-throughput approach. The sex-

linkage of ten putatively Y-linked genes identified by this method was not, however, maintained in dioecious *M. annua* individuals from wild populations (Table 3.1). For each of the ten genes sequenced in *M. annua* individuals from across the species' range, male and female individuals were found to share polymorphisms, with the sex-specific SNPs identified by the segregation analysis present in some female plants and absent in some males.

Table 3.1 Summary of the putatively sex-linked genes identified by segregation analysis of sequenced gene transcripts and tested in individuals from crosses and wild dioecious *M. annua* populations. All primers are shown in 5'-> 3' orientation, with forward primer (F) followed by reverse primer (R) in each case.

Gene	X- or Y-linked?	X/Y sequence divergence (%)	PCR primer sequences	Amplified region size (bp)	Sex-linked in crosses?	Sex-linked in wild individuals?
contig476	X	-	c476F2: TGATGACGAAGCTGAGGAC c476R1: ATCCATTTCATCACTCATGG	668	yes	-
contig91	Y	0.40	c91F1: TGGAATTCTCACACGACCTT c91R2: CCAAGTGAGTGGTGGGATAG	745	yes	no
contig1439	Y	1.07	c1439F1: TGTGGTTCAAGAACAGCAAA c1439R2: CTGTTAATGCAGCACCCAAT	1356	yes	no
contig4549	Y	1.06	c4549F1: CCCCTTCTGAAAGAGGAGAT c4549R1: GGGCAGTATCTGCTTACCAA	809	yes	no
contig5031	Y	1.44	c5031F2: GGCTTCTCACTTGAAATCCA c5031R2: CGAGCTCTGTTCCACTTCTC	660	yes	no
contig5282	Y	1.37	c5282F3: CATCAAAGTCCCAACGTTCT c5282R3: TCCTGCAAATTGAGAGGTTT	821	yes	no
contig8368	Y	1.80	c8368F1: ATTGTAACACCAACCGCACT c8368R1: AAGAACGCGAATGATGTTGT	494	-	no
contig8524	Y	2.37	c8524F4: CATGACTTGCCTGATCGAC c8524R1: CTTTGCTCAGCTTCTCCTTG	701	-	no
contig9125	Y	0.98	c9125F1: AGCTTCGAGAACTGAACGTG c9125R2: AGAAGGGTGAACGCACACTA	1886	-	no
contig9652	Y	1.03	c9625F2: TCAGAGATAAACCCGAACCA c9625R2: CACCACCACTCACCTCTCAT	377	-	no
contig10679	Y	1.86	c10679F1: TGGTGCCACTAATTTCCAAT c10679R4: GTTCGACAGGAACGGAGAC	721	-	no

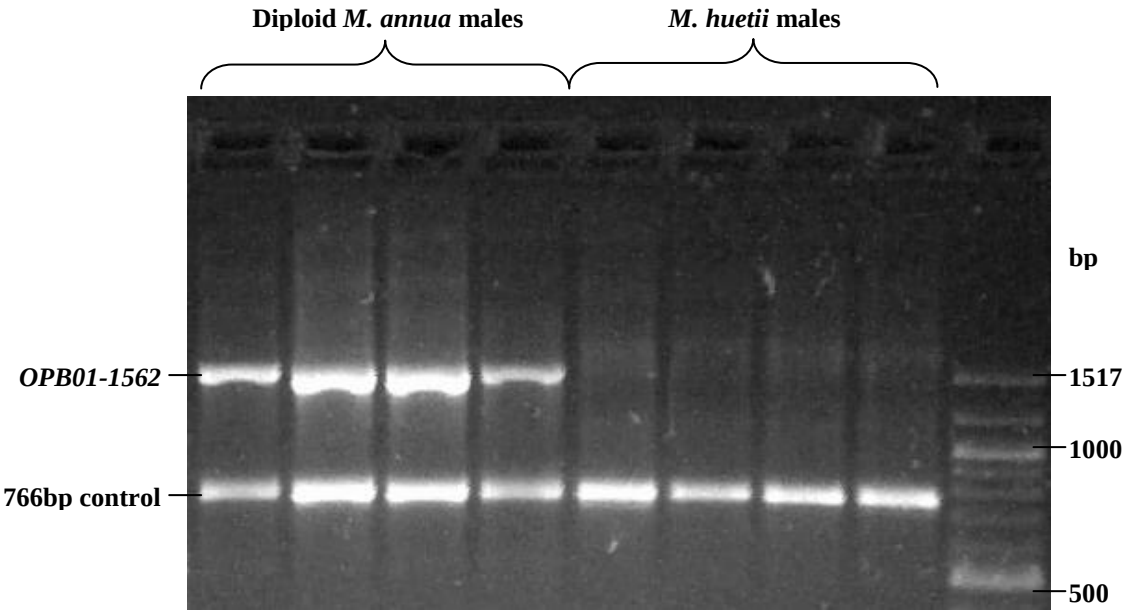


Figure 3.1 PCR amplification of *OPB01-1562* and a 766 bp control marker in four diploid *M. annua* males and four *M. huetii* males. Right hand lane contains fragment size calibration ladder (100bp DNA Ladder, New England Biolabs).

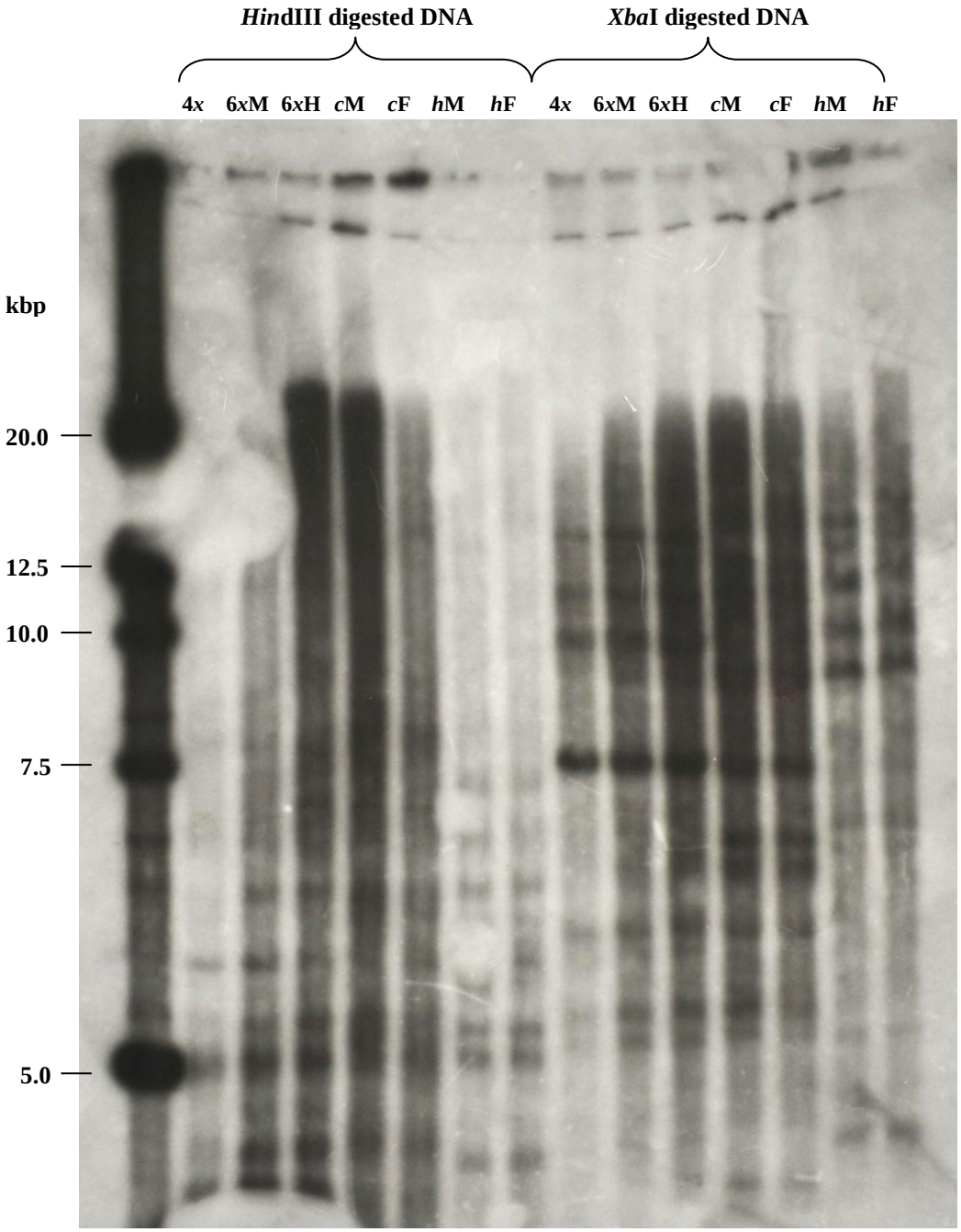


Figure 3.2 DNA blot of genomic DNA from *M. huetii*, *M. canariensis* and tetraploid and hexaploid *M. annua* individuals of separate sexes digested with *Hin*dIII and *Xba*I restriction enzymes and hybridised with *OPB01-1562*. Note the absence of sex-specific polymorphisms in all of the lineages and the multiple bands of strong signal obtained from all lineages. 4*x* = tetraploid *M. annua* DNA; 6*x* = hexaploid *M. annua*; *c* = *M. canariensis*; *h* = *M. huetii*; M = male; H = hermaphrodite; F = female. Left hand lane contains fragment size calibration ladder (GeneRuler 1kb Plus DNA Ladder, Fermentas).

3.4 DISCUSSION

3.4.1 Conservation of *OPB01-1562*

The results of both PCR amplification and DNA blot hybridisation analysis indicate *OPB01-1562* is not conserved as a sex-linked marker amongst lineages of annual mercuries other than diploid *M. annua*. The marker failed to amplify in all *M. huetii*, *M. canariensis*, tetraploid and hexaploid *M. annua* individuals tested by PCR, irrespective of gender, whilst no sex-specific polymorphisms could be detected with *OPB01-1562* in any of the lineages when the marker was used as a probe in DNA blot hybridisations. Interestingly, results from DNA blot hybridisations suggest regions of the marker may be present at multiple genomic locations in all of the lineages tested. This is perhaps not surprising given that internal regions of the marker, including the 766 bp control marker used in this study, amplify in female diploid *M. annua* as well as individuals of all sexes of other lineages. Furthermore, Khadka *et al.* (2005) reported that a 584 bp internal sequence derived from the 3' end of *OPB01-1562* shows homology to retrotransposons, with the translated sequence most similar to pineapple retrotransposon *dea1*. DNA blot hybridisations have suggested that this internal fragment, named *MARL* (*M. annua* retrotransposon like), which also amplifies in male and hermaphroditic hexaploid *M. annua* by PCR, is from a different locus to *OPB01-1562* (Khadka *et al.*, 2005). In contrast, the 766 bp control marker appears to be from the same locus, a point that may account for the absence of sex-specific hybridisations of *OPB01-1562* with diploid *M. annua* DNA, as the probe hybridised to the same genomic locations in females as well as males. It would thus seem that *OPB01-1562* is not a conserved sex-linked marker amongst lineages of annual mercuries other than diploid *M. annua*, and that

fragments of the marker are scattered through the genome of diploid *M. annua* and other annual mercury lineages.

The absence of *OPB01-1562* as a sex-linked marker in other lineages of dioecious annual mercury, particularly *M. huetii*, the sister species to diploid *M. annua*, is most likely attributable to genomic divergence between species at the *OPB01-1562* locus (perhaps influenced by the marker's potential capacity to self-transpose within the genome). The fact that *OPB01-1562* was not amplified by PCR in hexaploid male *M. annua* or male *M. huetii* does, however, leave open the possibility that the male-determining genotype of hexaploid *M. annua* was inherited from *M. huetii* rather than diploid *M. annua*. Were male-determining genes inherited from diploid *M. annua*, one may expect *OPB01-1562* to be present in hexaploid male individuals. Were male-determining genes inherited from *M. huetii*, however, one would not predict *OPB01-1562* to be sex-linked in hexaploids, given its absence in *M. huetii*. Nevertheless, alternative explanations for the origin of androdioecy remain plausible despite the absence of *OPB01-1562* in hexaploid male plants. For example, male-determining genes may have been derived from diploid *M. annua* via the hypothesised maternal progenitor of the hexaploid lineage, tetraploid *M. annua*, itself an autopolyploid believed to have arisen from duplication of the diploid *M. annua* genome (Figure 1.2; Obbard *et al.*, 2006b). Despite not being active in exclusively monoecious tetraploid populations, the male-determining genotype of diploid *M. annua* could have been reactivated in hexaploids, with linkage to *OPB01-1562* lost during polyploidisation, hybridisation and transitions in sexual system. Nonetheless, the identification of homologous sex-linked markers amongst lineages of the *M. annua* species complex, and *Mercurialis* species more widely, would be

highly informative for study of the evolution of androdioecy and monoecy in *M. annua* and of the conservation of sex-determining mechanisms across the genus.

3.4.2 Identifying new sex-linked genes in dioecious *M. annua*

The segregation analysis of sequenced gene transcripts from two crosses of dioecious *M. annua* identified a large number of putatively sex-linked genes. However, analysis of ten putatively Y-linked genes in dioecious *M. annua* sampled from across the species' range found that sex-linkage was not maintained in individuals from wild populations. These ten genes are thus probably located on the same chromosome pair as the genes for sex determination, but they are not tightly linked to the sex-determining locus, with recombination giving rise to shared polymorphisms between males and females in wild populations. In species with heteromorphic sex chromosomes, regions of the sex chromosomes in which recombination is not suppressed are often termed 'pseudoautosomal regions' (PARs; Otto *et al.*, 2011), and hence it could be said that these genes are located in a chromosomal region of *M. annua* analogous to the PAR of species with heteromorphic sex chromosomes.

Alternatively, it is possible that these genes are located in a non-recombining region around the sex-determining locus of *M. annua*, but that they have been incorporated in this region only relatively recently and hence X- and Y-linked homologues have not diverged significantly from one another. There could even be differences between populations in the boundary between non-recombining and recombining regions of the *M. annua* sex chromosome pair, with individuals tested from wild populations still recombining in the region in which the putatively Y-linked genes

are located, but recombination being suppressed in this region in those plants used in the segregation analysis. In either of these cases, the Y-linked variants identified in this analysis would be specific only to the parent plants or population used in the segregation analysis, being X-linked or absent in other wild individuals, and thus these genes are unsuitable for use as general sex-specific markers. However, these scenarios seem very unlikely; in the event of a non-recombining region having evolved in dioecious *M. annua*, one would still expect to see some divergence between X- and Y-linked homologues, and not the extent of shared polymorphism between males and females in all of the genes tested in this study. Furthermore, the sex-specificity of the putatively Y-linked genes was not maintained in any of the populations tested, including one located very close to the population from which plants were sampled for use in the segregation analysis, suggesting that population-level polymorphism in the boundary of a non-recombining region is unlikely to exist.

It is also possible that these putatively sex-linked genes are autosomal and that the co-segregation of SNPs with gender occurred purely by chance. Assuming no SNP-calling errors, the probability of a paternal polymorphism co-segregating exclusively with all individuals of one sex is approximately 1 in 500,000 ($0.5^{20} \times 2 = 1.91 \times 10^{-6}$). With 20,412 gene contigs identified during transcriptome assembly, the probability of one or more genes segregating thus is small (0.038), and the probability of selecting one or more such genes in a random sample of ten from the 464 putatively sex-linked genes identified is negligible (0.00082). As all SNPs were required to be covered by at least five sequence reads to avoid error, it is therefore

most likely that these results do not represent false positives and that these genes are at least partially linked to the sex-determining locus.

This work has hence been unable to identify any new fully sex-linked genes in dioecious *M. annua* for use in studying the conservation of sex determination and evolution of sexual systems in the annual mercury clade. Nonetheless, the segregation analysis described in this chapter represents an important first step in isolating such genes. Crosses between F_1 progeny obtained from this study are to be undertaken and the transcriptomes of F_2 progeny sequenced in order to identify those putatively sex-linked genes that still segregate with sex, thus eliminating a greater proportion of genes which are only partially sex-linked. Moreover, the identification of genes probably located on the sex-determining chromosome pair, but in regions in which recombination may still occur, presents exciting possibilities in itself for study of the evolution of sex determination. For example, the fact that none of the ten putatively Y-linked variants tested in wild dioecious *M. annua* plants were fully male-linked would suggest that the species either lacks a non-recombining region, or that any non-recombining region around sex-determining loci is relatively small. Segregation analyses of sequenced gene transcripts in *S. latifolia* identified many more putatively sex-linked genes than in *M. annua* (659 to 1,800 in *S. latifolia*; Bergero & Charlesworth, 2011; Chibalina & Filatov, 2011; Muyle *et al.*, 2012), with the sex-linkage of 12 out of 13 putatively Y-linked genes maintained in natural populations (Bergero & Charlesworth, 2011). In *S. latifolia* it therefore appears that the vast majority of putatively sex-linked genes identified by this technique are located in the non-recombining region of the sex chromosomes. This result is perhaps unsurprising given that recombination is thought to be suppressed over

much of the length of the sex chromosome pair of *S. latifolia* (Westergaard, 1958; Nicolas *et al.*, 2005; Marais *et al.*, 2008), a very different scenario to that emerging in *M. annua*. The absence of heterochromosomes in dioecious *M. annua* (Durand, 1963) also suggests that there has been little or no divergence between chromosomes on which sex-determining genes are located (unlike *S. latifolia*, in which the Y chromosome is at least partially degenerate; Marais *et al.*, 2008), further evidence that recombination is not suppressed over a large chromosomal region in dioecious *M. annua*. However, the exclusive co-segregation of *OPB01-1562* with male sex in a large number of plants tested in this thesis (Chapter 2) and in the work of Khadka *et al.* (2002; 2005), suggests that a degree of recombination suppression does exist in dioecious *M. annua*. Moreover, the sex-linkage of only a small proportion of the total number of putatively sex-linked genes identified by this study were tested in wild individuals, with a large number of genes still potentially located in a non-recombining chromosomal region. Further testing of these genes would thus be valuable to fully assess the extent of recombination suppression in the species.

Nevertheless, a small, or even absent, non-recombining region around the sex-determining loci of dioecious *M. annua* is perhaps surprising given that dioecy is assumed to be the ancestral condition in the annual mercury clade (and possibly in the *Mercurialis* genus as a whole), and that the sex-determining system of *M. annua* is hence thought to have been established for a significant period of evolutionary time. In a lineage with an established system of male heterogametic (XY) sex determination, one might predict an expanded region of recombination suppression, perhaps with a degree of chromosome degeneration, to have developed over time as sexually antagonistic genes accumulate around the sex-determining locus (see

section 2.1; Rice, 1987a; Charlesworth, 2002; Bergero & Charlesworth, 2009). Examination of ITS sequence variation (from Steinmann & Porter, 2002; Obbard *et al.*, 2006b) suggests dioecy may have arisen in a common ancestor of six *Mercurialis* species within the last 3 to 9 million years (assuming equal evolutionary rates in all lineages of Euphorbiaceae and using the dated phylogeny of Davis *et al.*; 2005), although this estimate should be treated cautiously given the assumptions made and the limited data available. Further study incorporating a greater number of sequences from a wider variety of lineages is necessary before confident estimates can be made of the age of dioecy in the clade. A recent analysis has also suggested that the genus *Mercurialis*, in which monoecy and dioecy are equivocal as ancestral states (Krähenbühl *et al.*, 2002), may be up to 66 million years old (Jovanović & Cvetković, 2010). In *Silene*, dioecy is estimated to have arisen between 5.6 and 9.5 million years ago (Rautenberg *et al.*, 2010; Marais *et al.*, 2011), with recombination estimated to have ceased between the sex chromosomes of *S. latifolia* between 5 and 10 million years ago (Bergero *et al.*, 2007), before the divergence of *S. latifolia* from its closest relatives (Marais *et al.*, 2011). Studies also indicate dioecy to be ancestral in the Cucurbitaceae family (Zhang *et al.*, 2006), implying a significant age for dioecy in this family, several members of which do possess heteromorphic sex chromosomes (e.g. *Coccinia indica* and *Trichosanthes dioica*; Kumar & Vishveshwaraiah, 1952; Patel, 1952; Sinha *et al.*, 2007). Dioecy is potentially younger in *M. annua* than *Silene* and Cucurbitaceae species with heteromorphic sex chromosomes, therefore, but more precise estimates of the age of separate sexes in the genus would be valuable for direct comparisons with other systems. The age of dioecy has also been estimated in very few other plant lineages in general, study of which would enable a more detailed examination of whether there is a correlation

between the age of separate sexes and the evolution of heteromorphic sex chromosomes (Charlesworth & Mank, 2010).

The absence of a large non-recombining region and degeneration in the sex chromosomes of *M. annua* is similar to the situation observed in numerous other dioecious plant species, however (e.g. *Asparagus officinalis, Spinacia oleracea* and *Fragaria virginiana*; Telgmann-Rauber *et al.*, 2007; Khattak *et al.*, 2006; Lan *et al.*, 2006; Spigler *et al.*, 2008), in which heteromorphic sex chromosomes are rare compared to animal groups (Charlesworth, 2002). Reasons for this rarity are unclear; although it is often attributed to the recent evolution of dioecy and subsequent young age of sex-determining systems in plant taxa, this is not necessarily correct as heteromorphic sex chromosome evolution may not be inevitable (Charlesworth, 2002; 2008). Alternatively, a low degree of selection for sexual dimorphism in plants has been hypothesised to limit the advantages of linking sexually antagonistic mutations to the sex-determining locus, thus precluding the expansion of regions of recombination suppression in plant systems, even in old dioecious lineages (Charlesworth, 2008; Charlesworth & Mank, 2010). Furthermore, it has also been suggested that autosomal genes may be important in controlling sexual dimorphism through sex-biased gene expression, as many sexually selected traits may be polygenic or controlled by genes with pleiotropic effects (Fry, 2009; Mank, 2009). Sexual dimorphism does exist in plants, however (Geber *et al.*, 1999), and a recent study has demonstrated that sex-linked quantitative trait loci (QTL) contribute heavily to sexual dimorphism in *F. virginiana*, a species with homomorphic sex chromosomes in which recombination suppression is not thought to have evolved (Spigler *et al.*, 2011). Spigler *et al.* (2011) detected the

presence of autosomal QTL for sexually dimorphic traits in *F. virginiana*, but sexual dimorphism was controlled almost exclusively by sex-linked QTL. Haploid expression of genes in male gametophytes has also been proposed to prevent chromosome degeneration in plants in order to maintain gene integrity on Y chromosomes (Charlesworth, 2008; Chibalina & Filatov, 2011). This does not explain, however, why heteromorphic sex chromosomes have evolved in some species (albeit a small number) in which haploid gene expression still occurs, or why large regions of recombination suppression have not developed in other dioecious species, with purifying selection preserving gene function, as has been conjectured in *S. latifolia* (Chibalina & Filatov, 2011). Why heteromorphic sex chromosomes have evolved in some dioecious plant species but not others thus remains a major puzzle in plant and evolutionary biology and *M. annua* may be a useful system for studying the early stages of sex chromosome evolution and examining the reasons for small regions of recombination suppression and reduced chromosome degeneration in dioecious plants relative to animals.

The identification of genes potentially located on the sex-determining chromosome pair of dioecious *M. annua*, but in regions in which recombination may still occur, also permits study of the PAR of the species. PARs are unique genomic regions, since despite exhibiting 'autosomal inheritance' (Blaschke & Rappold, 2006), their partial linkage to sex-determining loci (and consequent sex-biased inheritance of genes located within the PAR) has implications for selection on genes differentially influencing male and female fitness, and for genetic diversity (Otto *et al.*, 2011). I examine these questions in dioecious *M. annua* in the following chapter of this thesis.

4. THE DIVERSITY OF SEX-LINKED, PSEUDOAUTOSOMAL AND NON-SEX-LINKED DNA MARKERS IN DIOECIOUS *MERCURIALIS ANNUA*

4.1 INTRODUCTION

In the previous chapter, I sought to identify new sex-linked genes in dioecious *M. annua* through the use of a segregation analysis of sequenced gene transcripts. Analysis of ten putatively Y-linked genes isolated by this method found that sex-linkage was not maintained in individuals sampled from wild populations and that, although these genes are probably located on the same chromosome pair as the genes for sex determination, they are not located in a chromosomal region in which recombination is suppressed. This result suggested that dioecious *M. annua* has a small non-recombining region around the sex-determining loci, if a region of recombination suppression is even present at all (though the exclusive co-segregation of the DNA marker *OPB01-1562* with male sex suggests some degree of recombination suppression; Chapter 2). In this chapter, I extend my investigation by considering the patterns of genetic variation at these partially sex-linked loci, and compare estimated diversity levels to those of the fully male-linked marker, *OPB01-1562*, and putatively autosomal loci.

Many animal groups, including mammals and *Drosophila*, possess heteromorphic sex chromosomes (Bull, 1983). Such chromosomes are characterised by large regions of recombination suppression and genetic degeneration, with the loss of

most functional copies of genes on the Y chromosome, save those with functions specific to males (or degeneration of the W chromosome, save those with functions specific to females, in the case of female heterogamety; although X and Y notation will be used throughout this chapter, all statements are equally applicable to Z/W systems). Studies using sex-linked markers have also revealed low levels of genetic diversity in Y chromosomes relative to X chromosomes and autosomes of several animal groups, including mammals (Nachman, 1998; Hellborg & Ellegren, 2004), *Drosophila* (McAllister & Charlesworth, 1999; Bachtrog & Charlesworth, 2002) and birds (Montell *et al*., 2001). Heteromorphic sex chromosomes are rare in plant species, but the diversity of sex-linked versus non-sex-linked genes has been studied in *Silene latifolia* which possesses an at least partially degenerate Y chromosome (Marais *et al.*, 2008). Like animal groups, genetic diversity has been reported to be significantly lower in Y-linked genes compared to X-linked and autosomal genes of *S. latifolia* (Filatov *et al.*, 2000; 2001; Laporte *et al.*, 2005; Qiu *et al.*, 2010). Similar patterns in the genetic degeneration and diversity of sex chromosomes across a range of taxa suggest that similar processes may have acted during their evolution. Several mechanisms have been proposed to explain the low nucleotide diversity and degeneration of Y chromosomes, mechanisms which predominantly revolve around reductions in the effective population size and efficacy of selection in non-recombining chromosomal regions.

Genetic degeneration of Y chromosomes and the loss of genetic diversity have been largely attributed to population level processes reducing the effective population size (N_e) of the non-recombining Y region. N_e of Y-linked genes is expected to be lower than that of X-linked and autosomal genes as a consequence of the ploidy

differences between Y chromosomes and other chromosomes; all else being equal, N_e of Y-linked loci is supposed to be one quarter that of autosomal loci and one third that of X-linked loci, with N_e of X-linked genes being three quarters that of autosomal genes (simply put, in a diploid population with a 1:1 sex ratio there are four copies of autosomal genes and three copies of X-linked genes to every Y-linked gene; Caballero, 1995). Under neutrality, and assuming equal neutral mutation rates across all loci, therefore, one would predict Y-linked genes to possess proportionally less neutral diversity than X-linked and autosomal genes (all else being equal), even in sex chromosomes that are not degenerating. However, N_e of Y-linked non-recombining regions is reduced further by processes which also reduce the effectiveness of purifying selection, causing deleterious mutations to accumulate and leading to the gradual degeneration of the Y chromosome (Charlesworth & Charlesworth, 2000). These processes include: i) genetic hitchhiking – selective sweeps during the spread of favourable mutations lower diversity at linked loci and may lead to the fixation of deleterious alleles (Rice, 1987b; Bachtrog, 2004); ii) background selection – selection against strongly deleterious mutations reduces variation at linked neutral sites and causes accelerated stochastic accumulation of weakly deleterious alleles in remaining haplotypes (Charlesworth *et al.*, 1993; Kaiser & Charlesworth, 2009); iii) Muller's ratchet – the stochastic loss of Y chromosomes with the fewest deleterious mutations, exacerbated when N_e is small (Charlesworth, 1978; Gordo & Charlesworth, 2000; Gordo *et al.*, 2002; Engelstädter, 2008); and iv) sexual selection – high variance in male reproductive success may result in certain Y chromosomes contributing disproportionately to future generations and thus reduce N_e of Y chromosomes relative to X chromosomes (Caballero, 1995; Laporte & Charlesworth, 2002). The genetic diversity of Y-linked

regions is subsequently predicted to be reduced to a much greater extent than would be expected purely as a result of the ploidy differences between Y chromosomes and other chromosomes. Indeed, in animals, low levels of nucleotide diversity have been reported in the Y chromosomes of a range of taxa, summarised in Table 4.1a. Similarly, in *S. latifolia*, studies have indicated the diversity of Y-linked genes to be up to 30 times lower than those of X-linked and autosomal genes (Table 4.1b,c).

In addition to predictions about the genetic diversity of Y-linked regions, theory also makes several predictions about the diversity of regions partially linked to sex-determining loci, but in which recombination is not suppressed; in species with heteromorphic sex chromosomes, such regions are often referred to as 'pseudoautosomal regions' (PARs; Otto *et al.*, 2011). PARs are unique genomic regions; they are recombining, like autosomes, which maintains sequence homology and prevents genetic degeneration, but their linkage to sex-determining loci leads to the sex-biased inheritance of genes and has implications for selection on genes differentially influencing male and female fitness (sexually antagonistic genes; Otto *et al.*, 2011). For example, frequencies of sexually antagonistic alleles are predicted to diverge between X and Y chromosomes as a result of their sex-specific fitness effects and partial sex-linkage. Alleles advantageous in males but disadvantageous in females, for instance, are more likely to invade a population if linked to the Y-specific region, where the accumulation of such alleles may ultimately culminate in further recombination suppression and an expansion of the Y-specific region (see section 2.1; Rice, 1984; 1987a). If recombination rates are very low (due to tight linkage to the Y-specific non-recombining region, perhaps) then differences in allele frequencies of PARs can even accrue between X and Y chromosomes when alleles

have no sex-specific fitness effects as a result of overdominant selection (Clark, 1988). As alleles at PAR loci may spend different amounts of time on X or Y chromosomes, under overdominant selection an association can develop whereby one allele occurs more often in males and another occurs more often in females, thus increasing the likelihood of heterozygous offspring being produced (Otto *et al.*, 2011).

Further to implications for the inheritance of genes differentially influencing male and female fitness, linkage to the sex-determining region also has consequences for the genetic diversity of PARs. Diversity is predicted to be elevated between X and Y chromosomes in loci closely linked to the non-recombining region, but to decrease with increasing distance from the non-recombining region and converge with autosomal diversity levels as recombination rates rise (Kirkpatrick *et al.*, 2010; Otto *et al.*, 2011). Simulation studies by Kirkpatrick *et al.* (2010) also predict neutral diversity amongst X-linked loci to be elevated slightly relative to autosomal loci, whilst the diversity amongst Y-linked loci is expected to be reduced relative to autosomes. Differences in diversity are predicted to arise as a result of alleles spending different amounts of evolutionary time linked to X or Y chromosomes (for example, a predominantly Y-linked allele will be exposed to some degree to those processes which reduce the diversity of Y-specific regions, though to a much lesser extent; see above) and as a result of sexually antagonistic selection. For example, as female-benefit and male-benefit alleles become associated with X and Y chromosomes respectively, allele frequencies diverge between the sex chromosomes, leading to increased heterozygosity between X-linked and Y-linked alleles than amongst X-linked or Y-linked alleles alone (Otto *et al.*, 2011).

Moreover, mutation rates have also been reported to be significantly higher than the genomic average in pseudoautosomal genes of humans and apes, which may further elevate the diversity of PARs (Filatov & Gerrard, 2003; Filatov, 2004). Diversity levels of PARs may thus exceed those of autosomal loci, particularly in genes closely linked to the non-recombining region, where theory predicts it easier to maintain a sexually antagonistic polymorphism as a result of a reduction in recombination rate (although not if recombination is suppressed completely, as is the case in Y-specific regions, when diversity is predicted to decrease, as described above; Otto *et al.*, 2011).

The size of the PAR varies widely amongst taxa; in mammals, for example, the PAR is very small, with one PAR at each end of the sex chromosomes making up a total of 4.6% of the Y chromosome in humans (Rappold, 1993). In plants, however, PARs are generally much larger, comprising 83% of the chromosome pair in *Carica papaya* (Yu *et al.*, 2009), for example, and although precise estimates have been made in few other species, the single-locus mechanisms of sex determination identified in species such as *Asparagus officinalis* (Telgmann-Rauber *et al.*, 2007) and *Spinacia oleracea* (Khattak *et al.*, 2006; Lan *et al*., 2006) suggest very small non-recombining chromosomal regions and hence large PARs. Despite the attention paid to estimating nucleotide diversity in fully Y-linked versus X-linked and autosomal loci, few studies have estimated the diversity levels of PARs. Studies of the human PAR have produced varied estimates of diversity, with some similar to those of autosomal genes ($\pi = 0.00087$; May *et al.*, 2002) but others indicating much greater levels of diversity ($\pi = 0.0054$; Schiebel *et al.*, 2000; Bussell *et al.*, 2006). A study of diversity in the PAR of the emu, *Dromaius novaehollandiae*, reported that

nucleotide diversity was not significantly lower in pseudoautosomal loci ($\pi = 0.0019 \pm 0.0024$) than autosomal loci ($\pi = 0.0042 \pm 0.0061$; Janes *et al.*, 2009). Amongst plants, two putatively pseudoautosomal loci have recently been identified in *S. latifolia* in which diversity was estimated to be of a similar level to that of autosomal loci (Table 4.1c; Qiu *et al.*, 2010).

The identification of a fully male-linked DNA marker (see Chapter 2), and ten putatively partially sex-linked loci (see Chapter 3), in dioecious *Mercurialis annua* permits testing of the predictions for the genetic diversity of Y-linked markers and PARs outlined above. This is particularly interesting in *M. annua* given the apparent lack of degeneracy in the sex chromosomes of the species (Durand, 1963), and the inference that recombination suppression is probably restricted to a relatively small region around the sex-determining locus (see section 3.4.2). Many of the systems in which sex chromosomes and PARs have been studied extensively (including those cited above) possess highly heteromorphic sex chromosomes in which recombination is suppressed over most of the length of the sex chromosome pair and in which PARs are subsequently very small, or even non-existent. It is therefore interesting to consider the extent to which diversity differs between these different genomic regions in a species without heteromorphic sex chromosomes, and how this compares with the patterns of diversity reported in species with distinct sex chromosomes, including *S. latifolia*, a species with at an least partially degenerated Y chromosome (Marais *et al.*, 2008). In this chapter, therefore, I report sequencing and comparison of nucleotide diversity in the male-linked marker, *OPB01-1562*, and a selection of putatively pseudoautosomal and autosomal genes in dioecious *M. annua*.

Table 4.1a Summary of nucleotide diversity estimated in non-coding regions of X-linked (and Z-linked, in the case of avian taxa) and Y- (and W-) linked loci of various animal species.

Species	Estimated nucleotide diversity (π)		Reference
	X- or Z-linked loci	Y- or W-linked loci	
Homo sapiens (Human)	0.00137	0.00008	Nachman, 1998
Mus domesticus (Mouse)	0.00160	0.00041	Nachman, 1998
Lynx lynx (Lynx)	0.00016	0	Hellborg & Ellegren, 2004
Canis lupus (Wolf)	0.00038	0.00004	Hellborg & Ellegren, 2004
Microtus agrestis (Field vole)	0.00080	0.00017	Hellborg & Ellegren, 2004
Hirundo rustica (Barn swallow)	0.00139	0	Montell *et al*., 2001
Parus caeruleus (Blue tit)	0.00026	0	Montell *et al*., 2001
Falco tinnunculus (Kestrel)	0.00131	0	Montell *et al*., 2001
Drosophila miranda (Fruit fly)	0.00747	0	Bachtrog & Charlesworth, 2002

Table 4.1b Summary of silent site diversity estimated in X-linked and Y-linked gene pairs of *S. latifolia*.

X/Y Gene Pair	X-linked diversity		Y-linked diversity		Reference
	π	θ	π	θ	
SlX1/*SlY1*	0.0193	0.0200	0.0008	0.0007	Filatov *et al.*, 2001
SlX4/*SlY4*	0.0224	0.0383	0.0019	0.0014	Laporte *et al.*, 2005
DD44X/*DD44Y*	0.0218	0.0217	0.0016	0.0016	Laporte *et al.*, 2005
SlCypX/*SlCypY*	0.0088	0.0122	0.0007	0.0012	Qiu *et al.*, 2010
SlX7/*SlY7*	0.0222	0.0339	0.0015	0.0016	Qiu *et al.*, 2010
SlX9/*SlY9*	0.0415	0.0441	0.0021	0.0041	Qiu *et al.*, 2010

Table 4.1c Summary of silent site diversity estimated in autosomal and pseudoautosomal loci of *S. latifolia*. * Denotes pseudoautosomal loci.

Gene	Diversity		Reference
	π	**θ**	
CCLS37.1	0.0086	0.0093	Filatov *et al.*, 2001
E72	0.0281	0.0459	Qiu *et al.*, 2010
E106	0.0012	0.0063	Qiu *et al.*, 2010
E163	0.0193	0.0186	Qiu *et al.*, 2010
E265	0.0016	0.0022	Qiu *et al.*, 2010
E304	0.0101	0.0206	Qiu *et al.*, 2010
E314	0.0251	0.0250	Qiu *et al.*, 2010
E391	0.0680	0.0638	Qiu *et al.*, 2010
E393	0.0766	0.0730	Qiu *et al.*, 2010
*E241 **	0.0567	0.0673	Qiu *et al.*, 2010
*E284 **	0.0673	0.0709	Qiu *et al.*, 2010

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Seeds of diploid *M. annua* for this study were bulk collected from 13 wild populations across the species' natural range (Figure 4.1; Appendix 9.1). Approximately 50 seeds from each population were sown in soil-based compost in separate seed trays and grown under standard glasshouse conditions. After six weeks growth, once male plants were distinguishable from females by their pedunculate inflorescences, genomic DNA was extracted from fresh leaf material using a modified cetyltrimethylammonium bromide (CTAB) procedure (specific modifications are described in section 2.2.4; Doyle & Doyle, 1987). As sequencing of *OPB01-1562* was undertaken at a different time to that of putatively pseudoautosomal and autosomal genes, different individuals were sampled for sequencing of *OPB01-1562* than for other genes, although all individuals were sampled from similar geographic regions and, in some cases, the same populations (Figure 4.1). Specifically, DNA was extracted from two male and two female plants selected at random from each of the following six populations for sequencing of pseudoautosomal and non-sex-linked genes: 78a; 80a; 668b; 68a; J; and 228a (see Appendix 9.1 for full details of populations used). DNA was extracted from two to four male plants from each of the following ten populations for sequencing of *OPB01-1562*: 78a; 80a; 228a; RB; FT1; 65a; 70a; 1562a; 591a; and 579a.

4.2.2 DNA Sequencing

Sequencing of all markers was achieved by Sanger sequencing of polymerase chain reaction (PCR) products. All markers were amplified in all relevant individuals by PCR using reagents and amplification conditions identical to those described in

section 2.2.4. Primers for five non-sex-linked (putatively autosomal) genes, identified in dioecious *M. annua* by the segregation analysis of sequenced gene transcripts undertaken in Chapter 3 (see section 3.2.3), were designed using the program *Primer3* (Rozen & Skaletsky, 2000). The development of primers for ten putatively pseudoautosomal genes was completed in Chapter 3 (section 3.2.3) and five of these genes were randomly selected for sequencing in this study. *OPB01-1562* was amplified using primer pair B1F01/B1R01 (Khadka *et al.*, 2002). Full details of all markers sequenced and primer sequences are shown in Table 4.2. PCR products were visualized by ethidium bromide staining after electrophoresis on 1.5% agarose gels and were subsequently sequenced in both directions using BigDye terminator version 3.1 (Applied Biosystems), following the manufacturer's instructions. Internal primers were used in the sequencing of contig11, contig1439 and *OPB01-1562* in addition to terminal primers in order to achieve sufficient sequence coverage of these longer markers (Table 4.2). Sequences obtained for each individual were manually edited and assembled into contigs using *Sequencher* version 4.5 (Gene Codes). Consensus sequences from each individual were then aligned manually for each marker using *Se-Al* version 2.0a11 (http://tree.bio.ed.ac.uk/software/seal/) and assessed visually for any putative polymorphisms, all of which were confirmed by re-examination of the original sequence reads.

4.2.3 Analysis of DNA sequence variation

Haplotypes were reconstructed for all markers using the PHASE algorithm (Stephens *et al.*, 2001; Stephens & Donelly, 2003) in DnaSP version 5.10.01 (Librado & Rozas, 2009). Non-coding regions of putatively pseudoautosomal and

non-sex-linked genes were inferred by comparison of genomic DNA sequences with cDNA sequences from transcriptome sequencing undertaken as part of the segregation analysis (section 3.2.3). Reading frames of coding regions were assigned to sequences by *blastx* search of the cDNA of each gene against an *Arabidopsis thaliana* protein database (http://www.ncbi.nlm.nih.gov/BLAST/), and also by checking for in-frame stop codons using *ProSeq* version 3.2 (Filatov, 2009). Aligned and annotated haplotype sequences from all individuals for each gene were then imported into *ProSeq* for analysis.

DNA polymorphism was estimated for each marker using two standard measures: nucleotide diversity, π (Tajima, 1993); and Watterson's theta, θ (Watterson, 1984). Diversity per nucleotide estimates were calculated across all sites for each marker, as well as separately for non-coding regions and replacement and silent sites in coding regions, using *ProSeq* version 3.2 (Filatov, 2009). Insertion-deletions (indels) were treated as single polymorphisms in all cases. Diversity was compared between putatively pseudoautosomal and autosomal genes using Mann-Whitney *U*-tests, performed in *R*, version 2.8.0 (http://www.r-project.org). As the lengths of coding and non-coding regions differed between these genes, separate comparisons of diversity at silent sites and non-coding regions were made to compare the neutral genetic diversity of these gene types. The diversity of putatively pseudoautosomal genes at silent sites and non-coding regions was also compared between male and female *M. annua* individuals in order to assess whether there are differences in diversity between partially X-linked and partially Y-linked gene copies; as males possess both X- and Y-linked copies, theory predicts their diversity to be greater than that of females, which possess only X-linked copies (see section 4.1).

Nucleotide diversity (π; Tajima, 1993) of *OPB01-1562* across all base positions was compared against the diversity of putatively pseudoautosomal and non-sex-linked genes at all coding sites, silent sites and non-coding regions. Comparisons were made between the total diversity of *OPB01-1562* and diversity at all coding sites of other genes to ensure a conservative test of the hypothesis that the diversity of Y-linked markers is lower than that of non-sex-linked markers; as the coding nature of *OPB01-1562* is unknown, a simple polymorphism analysis in which the maximum amount of diversity in this Y-linked marker was compared against the regions of lowest diversity of other genes (coding regions were less variable than non-coding regions in all pseudoautosomal and non-sex-linked genes; see section 4.3.1) was deemed the most suitable approach.

Table 4.2 Summary of the non-sex-linked, pseudoautosomal and Y-linked DNA markers sequenced in dioecious *M. annua* individuals from across the species' natural range. All primers are shown in 5'-> 3' orientation, with forward primer (F) followed by reverse primer (R) in each case. * Denotes internal primers used in sequencing to achieve sufficient sequence coverage of longer markers. All primers for *OPB01-1562* were developed by Khadka *et al.* (2002).

Marker	**Marker Type**	**Marker length (bp)**	**PCR primer sequences**
contig1	Non-sex-linked	697	c1F2: CCTTGAGAGACGTGCAGAAT c1R1: AGATCGCTTCTCCAACTCAA
contig11	Non-sex-linked	1494	c11F2: CCCGACTCTTTTGACTTTGA c11R1: GATCCCTGTATTTGCACAGC c11F3: TATGGCATCGGAATACACCT * c11R3: TTCCACTCTCCTCTACTCTTCC *
contig28	Non-sex-linked	966	c28F2: CTACGGGGACAGGTTTTGTT c28R2: TGCTGCTGTTTTCTTTTCCT
contig29	Non-sex-linked	720	c29F1: TCACCCATTGCTTTCCTATC c29R2: AATCCTCCACAAACCTCACA
contig30	Non-sex-linked	848	c30F2: CGGTCTTATATCACCCGATG c30R1: CTGCTCCAGTCACCATCTTT
contig91	Pseudoautosomal	745	c91F1: TGGAATTCTCACACGACCTT c91R2: CCAAGTGAGTGGTGGGATAG
contig1439	Pseudoautosomal	1356	c1439F1: TGTGGTTCAAGAACAGCAAA c1439R2: CTGTTAATGCAGCACCCAAT c1439F2: CCATTCAAGGATGTTCCAAG * c1439R1: TCCCTTTCTCTTGGATCCTC *
contig4549	Pseudoautosomal	809	c4549F1: CCCCTTCTGAAAGAGGAGAT c4549R1: GGGCAGTATCTGCTTACCAA
contig5031	Pseudoautosomal	660	c5031F2: GGCTTCTCACTTGAAATCCA c5031R2: CGAGCTCTGTTCCACTTCTC
contig5282	Pseudoautosomal	821	c5282F3: CATCAAAGTCCCAACGTTCT c5282R3: TCCTGCAAATTGAGAGGTTT
OPB01-1562	Y-linked	1562	B1F01: GTTTCGCTCCAAGCACAAGT B1R01: GTTTCGCTCCATCAATATCTACC B1F03: CAAGGTTCTAATCAAGCGGGT * B1F04: ATGCAGTACTTGTATGGTGCGAC * B1R04: GAAAACAGAAACCTAAATCTATC * B1R06: CATAAGGTTCTTCAACATCAATAGC *

Figure 4.1 Map of Europe showing the population locations across the natural range of dioecious *M. annua* from which seeds were collected for use in the analysis of sequence variation of *OPB01-1562*, putatively pseudoautosomal and non-sex-linked genes. Map modified from *Wikipedia* (http://en.wikipedia.org/wiki/File:BlankMap-Europe_no_boundaries.svg).

4.3 RESULTS

4.3.1 Diversity of putatively pseudoautosomal versus non-sex-linked genes

No significant differences were detected in the diversity of putatively pseudoautosomal versus non-sex-linked genes at silent sites ($U_1 = 1.84$, $p = 0.175$; Figure 4.2) and in non-coding regions ($U_1 = 0.50$, $p = 0.480$). The diversity of putatively pseudoautosomal genes was also not significantly different between male and female individuals at either silent sites (Figure 4.3) or in non-coding regions. Diversity was lower in coding regions than non-coding regions of all pseudoautosomal and non-sex-linked genes, with the diversity estimates of all genes summarised in Table 4.3.

4.3.2 Diversity of the Y-linked marker, *OPB01-1562*

Sequencing of *OPB01-1562* in male diploid *M. annua* was complicated by the unexpected amplification of multiple copies of the marker in all individuals sampled. Across the length of the marker, 14 base positions were found to be polymorphic in all of the 29 individuals in which the marker was sequenced successfully. All individuals possessed the same pair of nucleotide bases at each of the 14 positions, indicating these to be fixed polymorphisms. Furthermore, sequence reads involving primers B1F01 and B1R06 were disrupted at position 182 by the same three base pair insertion in all individuals. Given: a) the exclusivity of *OPB01-1562* to male *M. annua* in all individuals tested during the course of this thesis, and by Khadka *et al.* (2002; 2005); and b) the absence of homozygotes for any of these 14 polymorphic base positions or indel in any of the individuals sequenced, I infer that there are at least two paralogous copies of *OPB01-1562* present in the Y-specific non-recombining region of dioecious *M. annua* which co-amplify during PCR.

In addition to the fixed polymorphisms described above, a further 11 base positions in *OPB01-1562* were found to be variable between individuals. Following the unexpected amplification of multiple copies of this marker, analysis of nucleotide diversity in *OPB01-1562* was subsequently modified as follows. First, the sequence obtained from each individual was separated into two copies using the PHASE algorithm (Stephens *et al.*, 2001; Stephens & Donelly, 2003) in DnaSP version 5.10.01 (Librado & Rozas, 2009). These two copies were then concatenated for each individual and analysed as a single marker. Since the precise copy number of *OPB01-1562* was unable to be ascertained from the sequencing data obtained in this study (although there were never more than two nucleotide bases per site at any of the polymorphic positions, one cannot rule out the possibility that there exists more than one copy of the marker with the same nucleotide bases at each of the polymorphic sites), an assumption of two copies was made in order to maintain a conservative test of the hypothesis that Y-linked marker diversity is lower than that of non-sex-linked markers. Assuming the minimum number of copies ensures that any variation in the marker is spread over the smallest number of possible sites, thus giving an estimate of the maximum amount of diversity in this Y-linked marker.

In summary therefore, *OPB01-1562* sequences showed 11 variable base positions amongst all individuals sequenced ($\pi = 0.00047 \pm 0.00049$; $\theta = 0.00100 \pm 0.00030$; Table 4.3). Diversity of *OPB01-1562* was lower than that of non-coding regions of all putatively pseudoautosomal and non-sex-linked genes, and was also lower than that of coding regions and silent site diversity of all genes except contig5031 (Figure 4.4).

Table 4.3 Summary of the diversity (± one standard deviation) in coding and non-coding regions of the non-sex-linked, pseudoautosomal and Y-linked markers sequenced.

Marker	Marker Type	Number of sequences	All sites				Coding regions, replacement sites			
			Sites analysed	Segregating sites	π	θ	Sites analysed	Segregating sites	π	θ
contig1	Non-sex-linked	46	642	15	0.0050 ± 0.0026	0.0053 ± 0.0014	421	7	0.0023 ± 0.0014	0.0029 ± 0.0011
contig11	Non-sex-linked	42	1412	58	0.0126 ± 0.0045	0.0094 ± 0.0012	258	2	0.0006 ± 0.0007	0.0015 ± 0.0010
contig28	Non-sex-linked	48	766	58	0.0177 ± 0.0067	0.0138 ± 0.0020	332	7	0.0049 ± 0.0017	0.0036 ± 0.0013
contig29	Non-sex-linked	48	696	39	0.0142 ± 0.0056	0.0117 ± 0.0019	537	17	0.0048 ± 0.0027	0.0055 ± 0.0013
contig30	Non-sex-linked	42	787	57	0.0140 ± 0.0079	0.0162 ± 0.0022	237	6	0.0036 ± 0.0022	0.0046 ± 0.0019
contig91	Pseudoautosomal	48	744	9	0.0019 ± 0.0013	0.0027 ± 0.0009	598	5	0.0009 ± 0.0007	0.0015 ± 0.0007
contig1439	Pseudoautosomal	48	1257	15	0.0039 ± 0.0013	0.0027 ± 0.0007	997	4	0.0007 ± 0.0004	0.0007 ± 0.0004
contig4549	Pseudoautosomal	48	785	32	0.0112 ± 0.0042	0.0086 ± 0.0016	563	7	0.0028 ± 0.0011	0.0022 ± 0.0008
contig5031	Pseudoautosomal	48	630	2	0.0006 ± 0.0004	0.0007 ± 0.0005	435	1	0.0003 ± 0.0002	0.0004 ± 0.0004
contig5282	Pseudoautosomal	48	780	75	0.0177 ± 0.0099	0.0205 ± 0.0024	453	20	0.0066 ± 0.0039	0.0080 ± 0.0018
OPB01-1562	Y-linked	29	2798	11	0.0005 ± 0.0005	0.0010 ± 0.0003	0	-	-	-

Table 4.3 (continued)

Marker	Marker Type	Number of sequences	Coding regions, silent sites				Non-coding regions			
			Sites analysed	Segregating sites	π	θ	Sites analysed	Segregating sites	π	θ
contig1	Non-sex-linked	46	122	4	0.0022 ± 0.0008	0.0017 ± 0.0008	99	4	0.0079 ± 0.0045	0.0092 ± 0.0046
contig11	Non-sex-linked	42	60	2	0.0016 ± 0.0007	0.0015 ± 0.0010	1094	54	0.0156 ± 0.0054	0.0113 ± 0.0016
contig28	Non-sex-linked	48	112	14	0.0096 ± 0.0031	0.0066 ± 0.0018	322	37	0.0221 ± 0.0094	0.0196 ± 0.0037
contig29	Non-sex-linked	48	159	22	0.0094 ± 0.0031	0.0065 ± 0.0015	0	-	-	-
contig30	Non-sex-linked	42	69	9	0.0057 ± 0.0033	0.0068 ± 0.0023	481	42	0.0169 ± 0.0093	0.0193 ± 0.0031
contig91	Pseudoautosomal	48	146	2	0.0009 ± 0.0003	0.0006 ± 0.0004	0	-	-	-
contig1439	Pseudoautosomal	48	258	11	0.0032 ± 0.0010	0.0020 ± 0.0006	0	-	-	-
contig4549	Pseudoautosomal	48	151	10	0.0035 ± 0.0015	0.0032 ± 0.0010	71	15	0.0613 ± 0.0199	0.0413 ± 0.0114
contig5031	Pseudoautosomal	48	114	0	0	0	81	1	0.0024 ± 0.0014	0.0028 ± 0.0028
contig5282	Pseudoautosomal	48	109	14	0.0035 ± 0.0027	0.0056 ± 0.0015	216	41	0.0375 ± 0.0191	0.0396 ± 0.0064
OPB01-1562	Y-linked	29	0	-	-	-	0	-	-	-

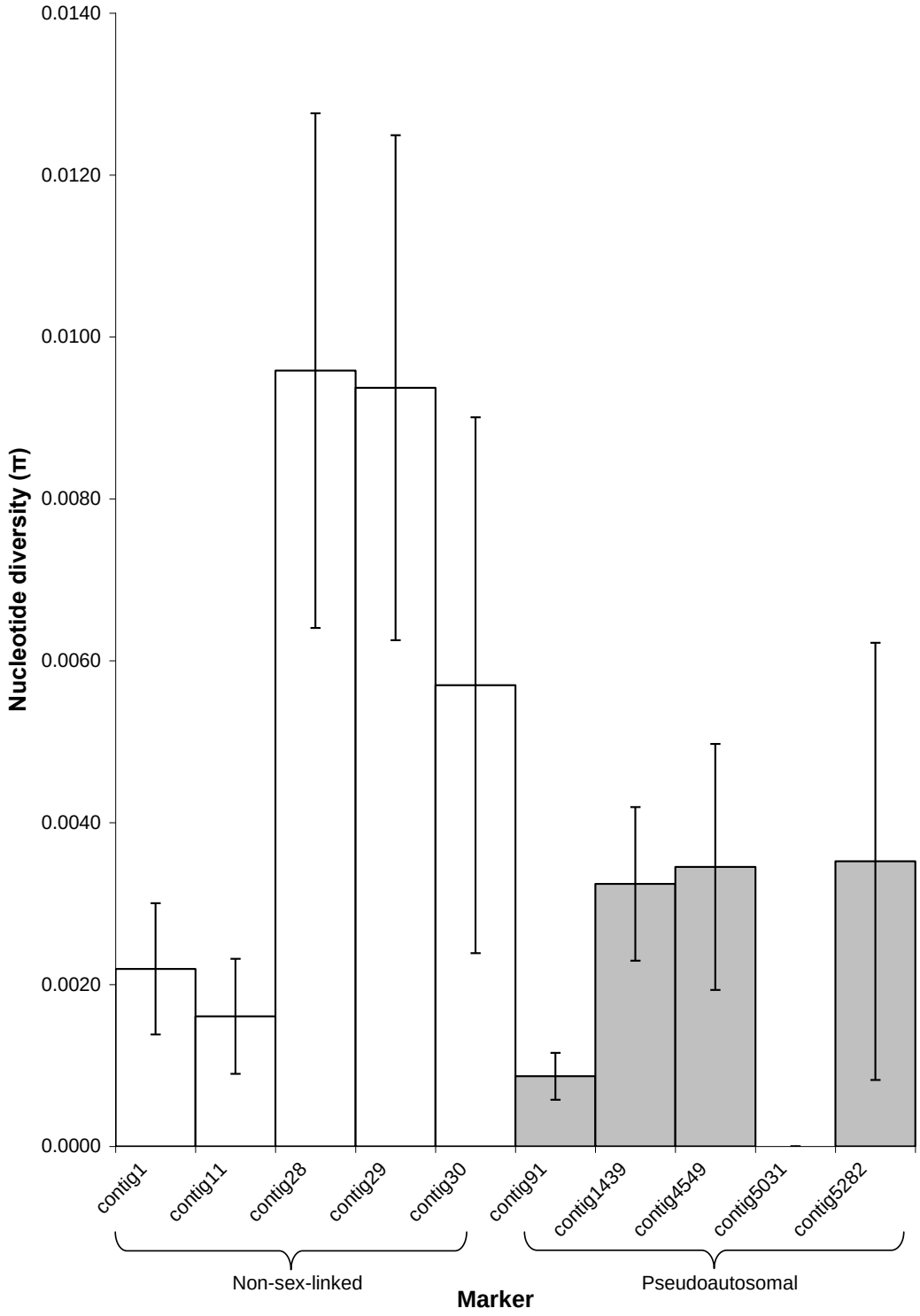


Figure 4.2 Estimated nucleotide diversity ($\pi \pm$ one standard deviation) of putatively non-sex-linked (white bars) and pseudoautosomal (grey) genes at silent sites in all individuals.

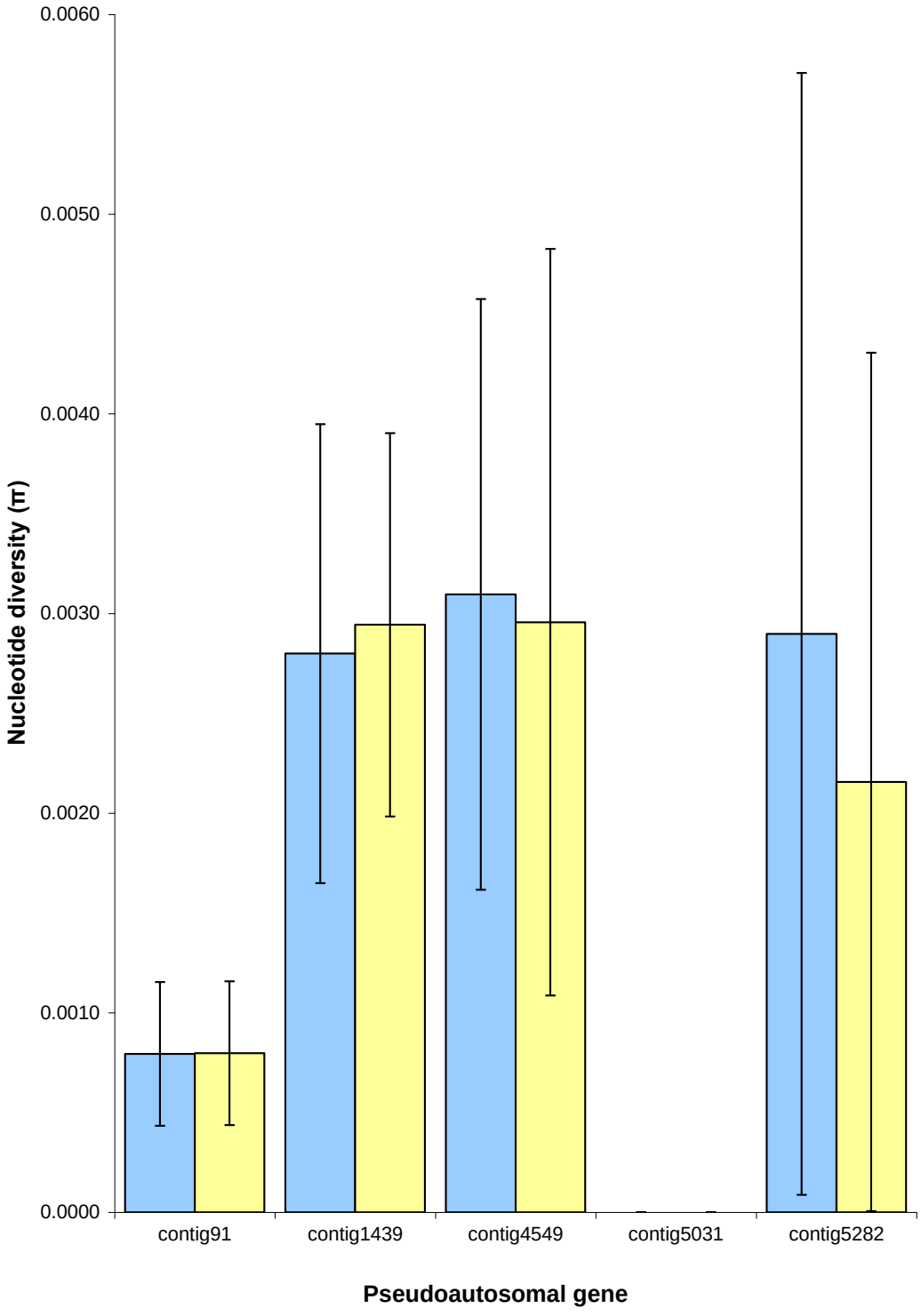


Figure 4.3 Estimated nucleotide diversity ($\pi \pm$ one standard deviation) of putatively pseudoautosomal genes in male (blue bars) and female (yellow) *M. annua* individuals at silent sites.

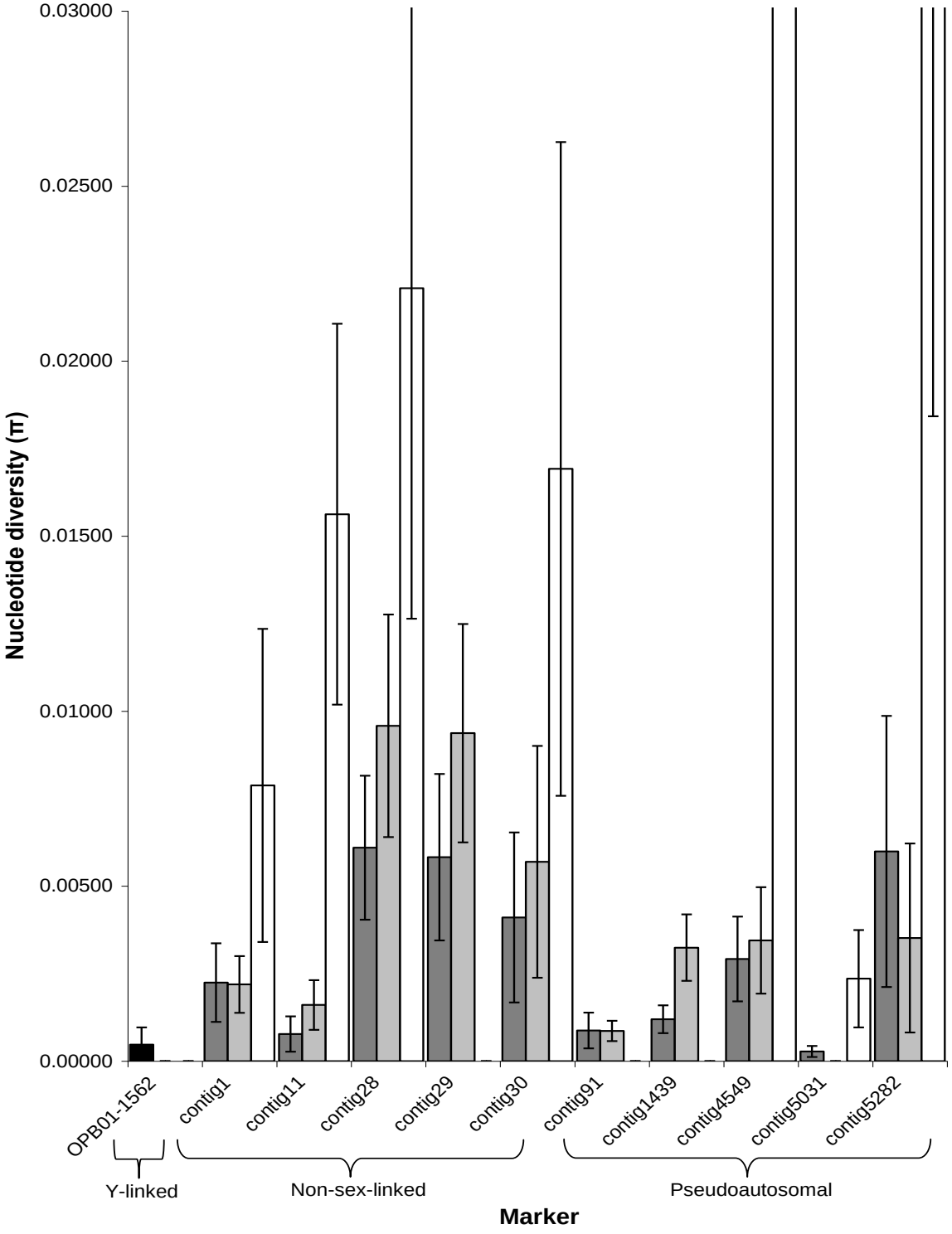


Figure 4.4 Estimated nucleotide diversity ($\pi \pm$ one standard deviation) of *OPB01-1562* (black bar) at all base positions and of putatively non-sex-linked and pseudoautosomal genes at all coding sites (dark grey), silent sites (light grey) and non-coding sites (white). The diversity of *OPB01-1562* is lower than that at coding sites, silent sites and non-coding regions of all putatively non-sex-linked and pseudoautosomal genes, with the exception of contig5031.

4.4 DISCUSSION

4.4.1 Diversity of putatively pseudoautosomal versus non-sex-linked genes

No significant difference was detected in the genetic diversity of putatively pseudoautosomal and non-sex-linked genes in dioecious *M. annua*. Furthermore, no difference was detected between the diversity of pseudoautosomal genes in male and female *M. annua* plants. These results do not, therefore, support the theoretical prediction that diversity is elevated between partially X- and partially Y-linked loci relative to autosomal loci as a result of alleles spending different amounts of evolutionary time on X and Y chromosomes (see section 4.1; Kirkpatrick *et al.*, 2010; Otto *et al.*, 2011). However, the most likely explanation for these findings is that the putatively pseudoautosomal loci used in this study are not sufficiently tightly linked to the supposed non-recombining chromosomal region in dioecious *M. annua* to facilitate the predicted increase in diversity levels. In addition to a predicted elevation in diversity, theory also predicts that diversity between partially X- and Y-linked loci will decrease with increasing distance from a non-recombining region. As recombination rates rise further from the Y-specific region, allele frequencies are prevented from diverging between the sex chromosomes and diversity levels are subsequently predicted to converge with those of autosomal loci. Given my inference in the previous chapter that the non-recombining region around the sex-determining locus in dioecious *M. annua* is probably relatively small (see section 3.4.2), and hence the PAR of the species is correspondingly large, it is perhaps unsurprising that pseudoautosomal genes may be located far from the sex-determining locus and that they experience recombination rates similar to those of autosomes. Interestingly, however, the diversity of two putatively pseudoautosomal loci in *S. latifolia*, a species with heteromorphic sex chromosomes and a small PAR,

has recently been estimated to be of a similar level to that of autosomal loci (Table 4.1c; Qiu *et al.*, 2010), whilst in animal taxa, studies of pseudoautosomal genetic diversity in species with heteromorphic sex chromosomes have produced variable results, some reporting elevated levels of diversity (e.g. Schiebel *et al.*, 2000; Bussell *et al.*, 2006), but others reporting diversity levels similar to those of autosomes (e.g. May *et al.*, 2002; Janes *et al.*, 2009). The results reported here likewise fail to match theoretical predictions and potentially suggest that additional factors may be influencing genetic diversity in pseudoautosomal loci; for example, extremely tight linkage, with recombination rates approaching zero, may be required to facilitate a divergence in allele frequencies between X and Y chromosomes and an increase in diversity. Further study of these unique genomic regions would therefore be extremely valuable to better understand the factors which influence their genetic diversity.

In dioecious *M. annua*, fine-scale mapping of putatively pseudoautosomal genes would be beneficial in order to estimate their distance from the sex-determining locus and address questions regarding the genetic diversity of the species' PAR. Since a large number of pseudoautosomal genes were potentially identified by the segregation analysis undertaken in Chapter 3, mapping of these genes would permit identification of those most closely linked to the non-recombining region, which may be more suitable for testing the theoretical predictions of genetic diversity in pseudoautosomal versus autosomal loci. Mapping would also enable testing of the prediction that the distance of pseudoautosomal genes from sex-determining loci influences genetic diversity. Moreover, the isolation of partially X- and partially Y-linked gene copies would permit a more precise estimate of differences in diversity

between and within these loci. The approach adopted here of comparing the diversity of pseudoautosomal genes between males (which possess both X- and Y-linked gene copies) and females (which possess just X-linked copies) is relatively weak, but isolation of alleles on X and Y chromosomes would improve this analysis.

An alternative explanation for the lack of significant difference in diversity between putatively pseudoautosomal and non-sex-linked loci is that the five pseudoautosomal loci tested here have no sex-specific fitness effects and are thus not subject to sexually antagonistic selection. In the absence of sexual antagonism, one would not predict allele frequencies to diverge between X and Y chromosomes (except under the rare scenario of overdominant selection and very low recombination; Clark, 1988), and thus no differences may arise in diversity relative to autosomal loci. A *blastx* search of the five putatively pseudoautosomal loci against a protein database indicates homology to the protein sequences of other plant species for all five genes, but provides no information on their likely function and whether they are involved in the regulation of sexually dimorphic traits. If mapping of the genes were undertaken and any were found to be relatively tightly linked to the non-recombining region, then this would provide support for this alternative explanation. In this event, it would be useful to assess the diversity of other genes located at a similar distance from the sex-determining region in order to establish whether all genes display similar levels of diversity, or whether some exhibit the predicted effects on diversity of partial linkage to the sex-determining region, which may indicate their involvement in the regulation of sexual dimorphism.

Alternatively again, if the putatively pseudoautosomal genes used in this study are actually located in the non-recombining sex-determining region of dioecious *M. annua* (which seems unlikely, but nevertheless possible; see section 3.4.2), then the detection of diversity levels similar to those of autosomal loci is somewhat surprising. In this case, the unexpectedly high diversity of these putatively sex-linked loci may be a result of their hypothesised recent assimilation into the non-recombining region, with insufficient evolutionary time having elapsed for X- and Y-linked homologues to diverge and for the predicted effects of suppressed recombination on genetic diversity to occur (see section 4.1). Once again, mapping to elucidate the precise location of these putatively pseudoautosomal genes would be informative for addressing this question.

Finally, the lack of significant difference detected in the diversity of pseudoautosomal versus non-sex-linked loci may be due to a lack of statistical power given the relatively small number of genes tested in this study. With the diversity of only five genes of each type estimated, it is possible that insufficient genes were sampled for differences in the diversity of these two gene types to be detected, i.e. that a Type II error has occurred due to insufficient sampling. For example, had one or more of the putatively pseudoautosomal genes tested in this study recently undergone a selective sweep, then diversity estimates of pseudoautosomal loci would consequently be reduced and, with only five pseudoautosomal genes tested, reduced diversity in just one gene would have a substantial effect on the overall estimate of pseudoautosomal diversity, which could potentially lead to differences in the diversity of putatively pseudoautosomal and non-sex-linked genes not being detected. This problem befell early investigations of

sex-linked versus non-sex-linked genetic diversity in *S. latifolia* (e.g. Filatov *et al.*, 2001), but may be overcome by increasing the number of genes tested, an option possible in dioecious *M. annua* given the large number of potentially pseudoautosomal and non-sex-linked genes identified by the segregation analysis undertaken in Chapter 3. Alternatively, however, rather than testing whether average diversity between putatively pseudoautosomal and non-sex-linked genes differs, given the data available from the segregation analysis, differences in diversity between these two genomic regions may be better assessed by examining the position of putatively pseudoautosomal loci on a distribution of the diversity of all non-sex-linked loci. 19,948 non-sex-linked genes were identified by the segregation analysis, with six copies of each of these genes sequenced as part of the analysis (two copies from each of the three parent plants used in crosses; see section 3.2.3). Using this data, one could estimate the diversity of each of these non-sex-linked genes and produce a distribution of putatively autosomal gene diversity, against which the diversity of putatively pseudoautosomal loci could be compared. One may thus be able to ascertain whether the diversity of putatively pseudoautosomal loci is indeed not significantly different to that of non-sex-linked loci (in which case putatively pseudoautosomal genes may be expected to be positioned randomly across a distribution of autosomal diversity), or whether there are actually differences in the diversity of putatively pseudoautosomal versus non-sex-linked genes which have not been detected in this study (in which case putatively pseudoautosomal loci may be expected to cluster in a non-random manner towards one of the tails of the distribution of autosomal diversity). Such an analysis may be a more powerful and appropriate test of differences in genetic diversity

between putatively pseudoautosomal and non-sex-linked genes in dioecious *M. annua*.

4.4.2 Diversity of the Y-linked marker, *OPB01-1562*

The nucleotide diversity of the Y-linked marker *OPB01-1562* was lower than that of non-coding regions of all putatively pseudoautosomal and non-sex-linked loci sequenced in this study, and was also lower than that of coding regions and silent site diversity of nine of the ten pseudoautosomal and non-sex-linked genes. However, the differences in diversity between *OPB01-1562* and other genes tested in this study are not generally lower than would be expected given the ploidy differences between Y chromosomes and autosomes. All else being equal, the N_e of Y-linked loci is one quarter that of autosomal loci (and of pseudoautosomal loci, assuming recombination rates in PARs are similar to those of autosomes; see above) and hence, under neutrality, neutral diversity of Y-linked regions is predicted to be one quarter that of autosomes (assuming equal neutral mutation rates across loci). Comparing the diversity of *OPB01-1562* to coding regions of pseudoautosomal and non-sex-linked loci, which is the most conservative comparison of diversity given that the coding nature of *OPB01-1562* is unknown and that coding regions of other genes are less diverse than non-coding regions, only five of the ten putatively pseudoautosomal and non-sex-linked genes have a diversity of more than four times greater than the Y-linked marker. Similarly, comparing the diversity of *OPB01-1562* to silent site diversity in other genes, just six of the ten putatively pseudoautosomal and non-sex-linked genes have a diversity more than four times greater than that of *OPB01-1562*. In *S. latifolia*, the diversity of Y-linked genes has been estimated to be 20 to 30 times lower than that of autosomal loci (Table 4.1b,c;

Laporte *et al.*, 2005; Qiu *et al.*, 2010), whilst exceptionally low levels of neutral diversity have also been reported in sex-linked loci in a range of animal species (Table 4.1a; Nachman, 1998; Montell *et al.*, 2001; Bachtrog & Charlesworth, 2002; Hellborg & Ellegren, 2004). A notable difference between these systems and *M. annua*, however, is the absence of a large non-recombining region and sex chromosome heteromorphism in *M. annua*. The hypothesised small region of suppressed recombination in the sex chromosome pair of *M. annua* may reflect a relatively recent origin of the sex-determining mechanism in the species, which may in turn account for the lack of a large difference between the diversity of Y-linked and autosomal and pseudoautosomal loci. However, as dioecy is assumed to be the ancestral condition in the annual mercury clade, and possibly in the *Mercurialis* genus, the sex-determining mechanism of *M. annua* has probably been established for a significant period of evolutionary time (and potentially a length of time not dissimilar to that of *S. latifolia*; see section 3.4.2), so the age argument is unlikely to explain the lack of extensive recombination suppression or the maintenance of genetic diversity in Y-linked regions in the species. Other explanations for the lack of extensive recombination suppression in *M. annua* and other dioecious plant species, including a lack of selection for sexual dimorphism in plants and the need to maintain gene integrity on Y chromosomes, are discussed in section 3.4.2, and it is plausible that the processes preventing expansion of the non-recombining region in dioecious plants are also responsible for maintaining genetic diversity in these genomic regions. Study of the genetic diversity of other dioecious species with small non-recombining regions would be useful for addressing this question.

Alternatively, it may be that *OPB01-1562* has been incorporated into a possible non-recombining region of the sex chromosome pair of *M. annua* only recently and that insufficient time has subsequently elapsed for genetic diversity to be reduced in the marker by processes reducing N_e of Y-linked regions. Given the marker's homology to retrotransposons, it is possible that it became sex-linked more recently than other Y-linked loci, and potentially after recombination suppression arose in the sex chromosome pair. In species with heteromorphic sex chromosomes, evidence indicates retrotransposons do accumulate in the male-specific region following the suppression of recombination (Sakamoto *et al.*, 2000; Charlesworth *et al.*, 2005; Ming *et al.*, 2011). On that note, it is also worth considering the likely coding nature of *OPB01-1562* and the most appropriate comparison of genetic diversity between this marker and the putatively pseudoautosomal and non-sex-linked loci sequenced in this study. Khadka *et al.* (2005) reported that although a 584 bp internal sequence derived from the 3' end of *OPB01-1562* shows homology to retrotransposons, it was interrupted by five stop codons and a frameshift relative to the retrotransposon sequences. This would suggest that *OPB01-1562* has lost its capacity to self-transpose and is static 'junk' DNA in the *M. annua* genome. In this case, a comparison between the genetic diversity of *OPB01-1562* and non-coding regions of pseudoautosomal and non-sex-linked loci would be the most appropriate test of diversity in sex-linked versus non-sex-linked regions. Interestingly, the diversity of non-coding regions of all seven pseudoautosomal and non-sex-linked genes which possessed non-coding DNA was at least five times greater than the diversity of *OPB01-1562*, meeting the theoretical prediction that Y-linked chromosomal regions are more genetically depauperate than non-sex-linked loci than would be expected due to ploidy differences alone. Furthermore, the diversity of *OPB01-1562* may

have been overestimated in this study as, in order to ensure a conservative comparison of diversity between sex-linked and non-sex-linked loci, there were assumed to be just two copies of the marker co-amplifying in the Y-specific region; in the event that more than two paralogous copies of *OPB01-1562* are Y-linked in *M. annua*, the estimated difference in diversity between sex-linked and non-sex-linked regions would be further increased. Further study of the coding nature and copy number of *OPB01-1562* would be useful to improve comparisons of the genetic diversity of this Y-linked marker with other non-sex-linked loci (see below).

In addition to further study of *OPB01-1562*, the identification of new sex-linked genes in dioecious *M. annua* would be extremely valuable to test theoretical predictions of genetic diversity in sex-linked versus autosomal loci. Isolating new fully and partially sex-linked genes would also allow further study of whether diversity levels of the Y-specific and pseudoautosomal regions of the *M. annua* genome are equivalent to those of autosomal loci. Moreover, the identification of active genes in dioecious *M. annua* (as attempted by the segregation analysis of gene transcripts undertaken in Chapter 3) would permit study of divergence between X- and Y-linked homologues and between other dioecious species in the *Mercurialis* genus, as well as an assessment of genetic degeneration and the conservation of gene function in Y-linked loci. With these resources, *M. annua* has further potential as a model system for studying sex chromosome evolution and the differences in genetic diversity between sex-linked and non-sex-linked loci.

4.4.3 Multiple, paralogous copies of the Y-linked marker, *OPB01-1562*

Sequencing of *OPB01-1562* in this study revealed the unexpected presence of multiple paralogous copies of the marker in the Y-specific non-recombining region of dioecious *M. annua*. In identifying the marker, Khadka *et al.* (2002) conducted a brief analysis of sequence variation in *OPB01-1562*, in which they identified six segregating nucleotide substitutions and a single base insertion amongst four male *M. annua* individuals. They did not, however, detect the presence of multiple copies of the marker. In contrast, 14 single base positions were found to be polymorphic in all 29 male plants in which the marker was sequenced in this investigation, whilst a three base pair insertion was also identified in at least one of the copies. These findings point towards the presence of multiple copies of *OPB01-1562* in a non-recombining, male-specific chromosomal region of dioecious *M. annua* for two reasons. First, in over three hundred individuals tested during the course of this thesis, *OPB01-1562* has been found to co-segregate exclusively with male sex in all plants, strongly indicating the marker to be hemizygous in males and linked to a dominant male-determining allele in a non-recombining region of the sex-determining chromosome pair of the species. Second, all 29 male plants in which *OPB01-1562* was sequenced in this study were heterozygous at all 14 aforementioned base positions; if the supposed polymorphism is due to heterozygosity at the *OPB01-1562* locus, then one would expect some homozygotes in a sample of this size.

The discovery of multiple paralogous copies of *OPB01-1562* is perhaps not surprising, however, given the reported homology of the marker to retrotransposons and the fact that fragments of the marker appear to be scattered through the genome of diploid *M. annua* and other annual mercury lineages (see Chapter 3).

Nevertheless, it would be helpful to elucidate the precise copy number of the marker in order to improve estimates of its genetic diversity. It would also be useful to determine how close the different copies are to one another in the putative Y-specific region in order to confirm the marker's hemizygosity and potentially estimate the size of the species' non-recombining region. Molecular cloning of *OPB01-1562* would be an effective way to ascertain the copy number of the marker, with the possibility of then designing paralogue-specific primers to amplify individual copies and, potentially, a larger fragment containing multiple copies of the marker and a larger portion of the Y-specific region.

4.4.4 Conclusions

The results of this study into the diversity of putatively sex-linked, pseudoautosomal and non-sex-linked DNA markers in dioecious *M. annua* have produced mixed results. No significant differences have been detected in the diversity of putatively pseudoautosomal and non-sex-linked loci, and whilst there is evidence that the diversity of the male-linked marker *OPB01-1562* is lower than that of other loci, it remains unclear whether diversity is lower than would be expected given the ploidy differences between Y-linked regions and autosomes. Fine-scale mapping of putatively pseudoautosomal loci to elucidate their chromosomal position relative to sex-determining loci and the non-recombining region of dioecious *M. annua* would be beneficial for addressing questions regarding the diversity of partially sex-linked loci. Furthermore, identification of new sex-linked genes in the species would also be a valuable resource for studying patterns of genetic diversity in sex-linked versus non-sex-linked loci, as well as for assessing the maintenance of gene integrity in a species lacking heteromorphic sex chromosomes. Dioecious *M. annua* is thus a

useful model for studying the evolution of sex chromosomes and the implications for genetic diversity of the evolution of sex-determining mechanisms.

5. THE EFFECTS OF POLYPLOIDISATION AND HYBRIDISATION ON SEX DETERMINATION AND SEX EXPRESSION IN THE ANNUAL MERCURIES

5.1 INTRODUCTION

Polyploidisation, the genome-wide multiplication of chromosome number, is a widespread phenomenon across eukaryotic taxa and an important factor in plant evolution (Ramsey & Schemske, 2002). Recent genomic analyses indicate polyploidy to be ubiquitous among angiosperms, with numerous incidences of genome duplication over the evolutionary history of flowering plants (Soltis *et al.*, 2009). Polyploidisation can be a source of novel variation (Otto & Whitton, 2000) and has been implicated in the evolution of fundamental biological traits, including sexual-system transitions (Pannell *et al.*, 2004), potentially via the disruption of sex-determining mechanisms (Westergaard, 1958).

Polyploidisation has long been acknowledged to disrupt sex determination. Using *Drosophila*, Muller (1925) showed that crosses of neo-tetraploids produced various sexual phenotypes (including sterile 'intersexes'), but not fertile tetraploids. In plants, artificially induced polyploids of dioecious *Silene* and *Rumex* were observed to produce some hermaphroditic progeny (reviewed in Westergaard, 1958). Indeed, the implications of polyploidy for sex-determining mechanisms have been proposed as an explanation for the rarity of polyploidy amongst animals relative to plants; assuming degenerate Y (or W, in the case of female heterogamety) chromosomes to

be a common feature of sex determination in animals but not plants, the disruption of dosage compensation following polyploidisation has been hypothesised to cause lethal imbalances in the products of genes found on X (or Z) chromosomes (Orr, 1990), although recent consideration of the problem has suggested this barrier is unlikely to account fully for the differences in polyploid occurrence between animals and plants (Mable, 2004).

Alternatively, polyploidisation may disrupt sex determination indirectly by precipitating changes in the sexual system of a species. For example, genome duplication may impact upon levels of inbreeding depression, floral morphology or self-incompatibility in a lineage, all key factors influencing sexual-system evolution (Pannell *et al.*, 2004). Were levels of inbreeding depression reduced in a neo-polyploid, for instance, conditions may favour the evolution of hermaphroditism in what was formerly a dioecious lineage, potentially with selection for increased selfing and reproductive assurance (which is also likely to improve the probability of a new polyploid lineage establishing; Levin, 1975; Rausch & Morgan, 2005). On the other hand, it is widely recognized that polyploidisation can cause gametophytic self-incompatibility systems to breakdown (Lewis, 1960; Stone, 2002) and it has been suggested that dioecy has often evolved as a response to selection for outcrossing following such a breakdown (Miller & Venable, 2000). Nonetheless, any transition in sexual system would necessitate the evolution of a new, or modification of an existing, mechanism of sex determination, and thus polyploidisation may also indirectly influence the evolution of sex determination in a lineage.

Multiple pathways to polyploidy have been proposed in plants (reviewed in Ramsey & Schemske, 1998), with the two most common mechanisms of polyploidisation involving the somatic doubling of cells or the fusion of unreduced (2*n*) gametes. Polyploidisation in angiosperms is also often associated with hybridisation. Genome duplication provides hybrids with an escape from low fertility, as viable gamete production is inhibited by differences in chromosome number and structure, preventing pairing and segregation during meiosis (Sybenga, 1975; Ramsey & Schemske, 1998). Polyploids formed as a result of hybridisation are referred to as 'allopolyploids', whilst polyploids which arise as the result of genome multiplication within a single species are termed 'autopolyploids'. The coming together of previously divergent genomes via hybridisation presents interesting questions with regard to sex determination in allopolyploids. If parental lineages possess diverse systems of sex determination, for example, what will be the adopted mechanism in a novel polyploid genetic background?

Polyploidisation and hybridisation are both believed to have played important roles in the evolution of the *Mercurialis annua* species complex (Obbard *et al*., 2006b). Across the species' geographic range, populations display variation in ploidy, from diploid to 12-ploid. Recent phylogenetic analyses indicate diploid, tetraploid and hexaploid lineages of *M. annua*, together with the sister species *M. huetii*, form a single clade of weedy annual mercuries (Figure 1.2). Molecular evidence indicates tetraploid *M. annua* to be of autopolyploid origin, arising from duplication of the diploid *M. annua* genome. Hexaploid *M. annua* is supposed to be an allopolyploid hybrid of tetraploid *M. annua* and *M. huetii*, the putative maternal and paternal progenitors respectively (Figure 1.2, Obbard *et al*., 2006b). Interestingly, sexual

system varies with ploidy in the clade; diploid *M. annua* and *M. huetii* are both dioecious, whereas tetraploid *M. annua* populations are exclusively monoecious and hexaploid *M. annua* populations are either monoecious or androdioecious, a rare breeding system consisting of males coexisting with functional (monoecious) hermaphrodites. This variation suggests that polyploidisation and hybridisation have had major impacts on the evolutionary trajectory of lineages of annual mercuries and on their systems of sex determination.

As dioecy is believed to be the ancestral state in the annual mercury clade (see section 3.1; Durand & Durand, 1992; Krähenbühl *et al*., 2002; Obbard *et al*., 2006b), the group provides an example of the evolution of functional hermaphroditism (monoecy) from separate sexes (dioecy) with polyploidisation. Several theories have been put forward purporting to explain the evolution of monoecy and androdioecy in polyploid *M. annua* lineages. With regard to the evolution of monoecy in tetraploid *M. annua*, autopolyploidisation itself may have directly triggered the loss of unisexuality by disrupting the sex-determining mechanism of dioecious *M. annua* (Pannell *et al*., 2004). For example, as discussed above, genome duplication may disturb sex-determining systems in which relative allele dosage is important, culminating in the segregation of a monoecious phenotype in neo-tetraploids, with no direct selection for functional hermaphroditism necessarily having occurred (Westergaard, 1958; Pannell *et al*., 2004). Indeed, Durand (1963) observed monoecious individuals to segregate in the F_2 progeny of artificially induced neo-tetraploids generated from diploid dioecious *M. annua*. Alternatively, the emergence of a monoecious phenotype following polyploidisation may have led to selection for functional hermaphroditism for several reasons. For example, an

ability to self-fertilise would enable neo-polyploids to avoid the fitness costs associated with back-crossing with diploid progenitors (Levin, 1975; Rodriguez, 1996; Rausch & Morgan, 2005). Neo-polyploids are likely to arise initially at exceptionally low density, and the capacity to self would thus be advantageous to avoid the production of sterile mixed ploidy hybrids and overcome the phenomenon of 'minority cytotype exclusion' (Levin, 1975), whilst it would also provide reproductive assurance when colonising new areas of marginal habitat (Brochmann *et al*., 2004; Pannell *et al*., 2004). Alternatively again, polyploidisation may reduce levels of inbreeding depression in a lineage below the threshold at which separate sexes becomes an advantageous trait and thus hermaphroditism may have been actively selected for over dioecy (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987). One of the major theories accounting for the evolution of unisexuality is as a consequence of selection for the benefits of inbreeding avoidance (Lloyd, 1975; Charlesworth & Charlesworth, 1978a), but if polyploidisation were to cause a significant decrease in the fitness costs associated with inbreeding, then functional hermaphroditism may become the superior sexual strategy. In any event, the evolution of monoecy in tetraploid *M. annua* following the genome duplication of diploid dioecious *M. annua* is likely to have impacted upon the sex-determining mechanism of the lineage, whether directly or as the result of selection for combined over separate sexes.

The origin of androdioecy in hexaploid lineages of *M. annua* is particularly interesting given the theoretical prediction that androdioecy should be much harder to evolve from hermaphroditism than dioecy or gynodioecy (Lloyd, 1975; Charlesworth & Charlesworth, 1978a; Charlesworth, 1984; Pannell, 2002). In order

for males to coexist with functional hermaphrodites, they must be at least twice as fertile as the male reproductive function of the hermaphrodites, a factor which increases with increasing selfing rates amongst the hermaphrodites. Since hexaploid *M. annua* has been proposed to be of allopolyploid origin, it has been suggested that androdioecy evolved in the lineage as a direct result of hybridisation (Obbard *et al.*, 2006b). Rather than evolving by the spread of a female sterility mutation in a hermaphroditic population (and facing the somewhat prohibitive requirements cited above), it has been conjectured that androdioecy arose as a result of the inheritance of a fully functional male-determining genotype from *M. huetii*, the putative paternal progenitor of this allohexaploid. Given the morphological similarity of males across the annual mercury clade (all males bear staminate flowers on erect axillary peduncles), it seems plausible that genes for a specialised male phenotype were inherited directly from male *M. huetii* rather than evolving *de novo* in neo-hexaploids (Obbard *et al.*, 2006b; although note that this hypothesis does assume functional conservation of a male-determining genotype between *M. huetii* and polyploid *M. annua*). Similarly, genes for a monoecious phenotype in hexaploid *M. annua* are hypothesised to have been inherited from the putative maternal progenitor, tetraploid *M. annua*.

Alternative explanations for the evolution of androdioecy in hexaploid *M. annua* also exist. Since tetraploid *M. annua* is hypothesised to be of autopolyploid origin from diploid *M. annua*, it is possible that male-determining genes are derived from the maternal progenitor and are originally of dioecious *M. annua* origin. Such genes are not active in tetraploid populations, hence all tetraploids populations are monoecious, but they have been reactivated in hexaploids, leading to androdioecy.

It is also possible that androdioecy evolved in hexaploid *M. annua* from dioecy, with selection on females to produce some pollen, perhaps for reproductive assurance under mate or pollen limitation, such as during colonisation (Pannell, 2001). Androdioecy in *Datisca glomerata*, for example, is proposed to have evolved from dioecy following the spread of a mutation that allowed females to produce pollen (Wolf *et al.*, 2001), and this pathway has also been suggested for the evolution of androdioecy in the small number of other plant species in which it has been reported, including *Spinifex littoreus* (Connor, 1996), *Schizopepon bryoniaefolius* (Akimoto *et al.*, 1999) and *Castilla elastica* (Sakai, 2001). This hypothesis seems less likely in *M. annua*, however, given the absence of dioecy in all other polyploid *M. annua* lineages. Nevertheless, modification of the sex-determining mechanism of hexaploid *M. annua*, relative to that of its progenitors, has evidently occurred in order to produce an androdioecious mating system. In this chapter, I thus explore the role that polyploidisation and hybridisation have played in precipitating these modifications in polyploid *M. annua*.

In addition to the effects of polyploidisation and hybridisation on sex determination in allopolyploids, hybridisation alone can also impact upon sex determination. If two species with different mechanisms of sex determination, or even different sexual systems, are hybridised, for example, how will gender be assigned in hybrids? Study of the sex expression of hybrids can be informative for assessing the conservation of sex-determining mechanisms and functionality of sex-determining genes between divergent lineages. For example, reciprocal crosses conducted between androdioecious *D. glomerata* and its dioecious sister species *D. cannabina* yielded only male and female hybrid progeny, from which it was inferred that genes

conferring hermaphroditism are recessive to those for unisexuality and that hermaphroditism evolved from dioecy in *D. glomerata* via spread of a recessive mutation for hermaphroditism (Wolf *et al.*, 2001). Similarly, crosses between *Silene latifolia* and *S. dioica* indicate that active-Y sex determination and the genetic basis of sex ratio distortion is conserved between these species (Taylor, 1994b), an inference which has since been corroborated by molecular data, with the Y chromosome believed to have arisen before the divergence of the two species (Mrackova *et al*., 2008; Marais *et al.*, 2011). With regard to annual mercuries, how are the mechanisms of sex determination of the various lineages related to one another? In Chapter 3 of this thesis, sex-linkage of a DNA marker from dioecious *M. annua* was found not to be maintained in other lineages of annual mercuries, a result most likely attributable to genomic divergence between species, although it could potentially suggest modification of the sex-determining mechanism between lineages, or even multiple independent origins of sex-determining mechanisms in the genus. Moreover, is the functionality of sex-determining genes conserved between lineages? The evolution of dioecy in plants is hypothesised to involve the establishment of both male- and female-sterility mutations (see section 2.1; Charlesworth & Charlesworth, 1978a); have these mutations been modified or suppressed in monoecious polyploid lineages? And are the sterility genes of dioecious lineages still functional in polyploid backgrounds? For example, will the dominant male-determining allele of dioecious *M. annua* (Chapter 2) still give rise to males in a polyploid background, or will genes for female function in polyploid, hermaphroditic *M. annua* be dominant over those for female-sterility from the dioecious system and restore female function? In this case, will female function be completely, or only partially, restored? These questions are addressed in this study

through an examination of the sex expression, reproductive effort and sex allocation of hybrids of various annual mercury lineages.

Finally, hybridisation can also impact upon the fertility and morphology of individuals relative to their progenitors. Differences in chromosome number and structure between parent lineages often prevent viable gamete production in hybrids, as chromosomes are unable to pair and segregate properly during meiosis (Sybenga, 1975), an important mechanism of post-zygotic reproductive isolation that maintains species identity in polyploid plants (Ramsey & Schemske, 1998). Amongst annual mercuries, differences in ploidy between lineages may therefore be predicted to significantly reduce the fertility of hybrids. Indeed, hybrid sterility has been implicated in the movement of the diploid-hexaploid *M. annua* contact zones in Spain, with pollen swamping by diploid plants causing a substantial proportion of hexaploid progeny to be tetraploid hybrids of low fertility, thus severely compromising the reproductive potential of hexaploids and causing them to be displaced by diploids (Buggs & Pannell, 2006). Hybrid fertility is thus important in shaping the geographic distribution of annual mercury lineages and in maintaining lineage identity and I therefore examine the fertility of the hybrids generated in this study. Furthermore, the range of differences in genome size amongst lineages presents an opportunity to test whether this influences hybrid fertility in annual mercuries; i.e. are hybrids of diploid and tetraploid *M. annua* more or less fertile than those of diploid and hexaploid *M. annua*, for example?

With regard to morphology, hybrid morphology may be predicted to be intermediate between that of parental lineages (Rieseberg & Ellstrand, 1993), but interactions

between parental genomes can also result in hybrids displaying more extreme morphological traits than either parent, a phenomenon known as transgressive segregation (Lexer *et al.*, 2003; Rieseberg *et al.*, 2003a). Hybridisation can thus be an important source of variation (Rieseberg *et al.*, 2003b; 2007) and it is interesting to consider the extent to which hybrids of various lineages of annual mercuries display intermediate, or more extreme, morphological variation relative to their progenitors. I therefore also undertake a comparative morphological analysis of annual mercury hybrids and their parent lineages to examine the effects of hybridisation on plant morphology.

In this chapter, therefore, I study the direct effects of polyploidisation and hybridisation on sex determination, sex expression, fertility and morphology of annual mercuries using two approaches. First, I hybridise various lineages of the annual mercury clade through controlled reciprocal crosses and analyse the sex ratios, reproductive effort, sex allocation and fertility of the hybrids produced. I also perform linear discriminant function analyses to examine the effects of hybridisation on plant morphology and whether hybrids are distinguishable from their parent lineages by any specific morphological characters. Second, I aim to reconstruct the hypothesised evolutionary history of the annual mercury clade by artificially recreating the putative polyploidisation events which led to the formation of tetraploid and hexaploid *M. annua*. Through colchicine treatment of germinating seeds, I generate neo-polyploids of diploid *M. annua* and *M. huetii* x tetraploid *M. annua* hybrids in which I examine sex determination and sex expression. Colchicine is a micro-tubule depolymerising agent which inhibits mitosis and is widely used for inducing polyploidy in plants (Dewey, 1980). For example, it has been used to

artificially reconstruct polyploid schemes in a number of plants for a variety of studies, including: gene loss and genome evolution in *Tragopogon* (Buggs *et al.*, 2009; Tate *et al.*, 2009; Malinska *et al.*, 2011); genomic structural rearrangements in *Brassica* (Gaeta & Pires, 2010; Xiong *et al.*, 2011); and gene expression in *Brassica* (Gaeta *et al.*, 2007; 2009), *Senecio* (Hegarty *et al.*, 2005; 2006; 2011) and *Arabidopsis* (Madlung *et al.*, 2005; Wang *et al.*, 2006). However, artificial reconstruction of a polyploid scheme to study the effects on sex determination and sex expression in a scheme in which sexual system also varies with ploidy has not been undertaken previously. I thus use this method to examine the effects of neo-polyploidisation on sex determination in *M. annua* and on how this may have contributed to shifts in the sexual system within the *M. annua* species complex, as outlined above. In particular, I explore whether genome duplication could have triggered the development of a monoecious phenotype directly, or whether selection for hermaphroditism in neo-polyploids must also have been involved.

5.2 MATERIALS AND METHODS

5.2.1 Annual mercury hybrids

Hybrids of various lineages of the annual mercury clade were generated by artificial, reciprocal crosses. Seeds of *M. huetii* and diploid, tetraploid and hexaploid (androdioecious) *M. annua* were bulk collected from a single population of known ploidy for each lineage (ploidy previously assessed by Obbard *et al*., 2006b; see Appendix 9.1 for full details of populations used). Approximately 100 seeds from each population were sown in soil-based compost in separate seed trays and grown under standard glasshouse conditions. After four weeks' growth, following germination and flowering, a random sample of individuals from each population were transplanted into individual 15 cm diameter pots of soil-based compost to be used in crosses. Specifically, the following numbers of individuals of each gender were transplanted out for each lineage: 20 female and 20 male *M. huetii*; 40 female and 30 male diploid *M. annua*; 40 monoecious tetraploid *M. annua*; and 10 male and 20 monoecious hexaploid *M. annua*.

Once the transplants were established, crosses between the lineages were undertaken. Four hybridisations were performed, namely: diploid *M. annua* x *M. huetii*; tetraploid *M. annua* x *M. huetii*; diploid *M. annua* x tetraploid *M. annua*; and diploid *M. annua* x hexaploid *M. annua*. Each cross was performed in both directions, i.e. using each lineage as both the male and female parent in the cross. In the case of the diploid *M. annua* x hexaploid *M. annua* hybridisation, hexaploid male plants and hexaploid monoecious plants were separately used as the father, thus giving a total of nine separate crosses performed. A summary of the crosses performed and the relationships between the lineages used is shown in Figure 5.1.

To conduct crosses, nine isolated glasshouses in the Department of Plant Sciences, University of Oxford, each free of any other *Mercurialis* plants, were used over winter 2010-2011. One cross was assigned to each glasshouse, with ten plants of the maternal lineage for the hybridisation and ten plants of the paternal lineage being randomly positioned on the glasshouse bench amongst one another. All plants were watered and maintained for a period of ten weeks to allow open pollination of the maternal plants by the paternal lineage. In crosses where monoecious tetraploid or hexaploid *M. annua* were used as the maternal parent, these individuals had their male flowers carefully removed (emasculation) using tweezers every three days to prevent pollen contamination. After ten weeks' growth, seeds from all ten maternal plants per cross were collected.

The seeds obtained from each of the ten maternal plants per cross were subsequently mixed to create bulk seed stocks for each hybridisation, before a random sample were sown in soil-based compost in seed trays and grown under standard glasshouse conditions. Upon flowering, hybridisation success, measured as the percentage of hybrids out of total progeny produced, was assessed for each cross. In the case of diploid *M. annua* x *M. huetii* crosses, hybridisation success could be visually assessed as hybrids were clearly distinguishable from their parent lineages by their intermediate morphology (see section 5.3.4). For the other seven crosses (all of which involved crosses between individuals of different ploidy levels), a random selection of up to 20 progeny of each gender per cross were assessed for hybridisation success by estimating their ploidy levels using flow cytometry. For each plant, approximately 15 mg of leaf material together with approximately 15 mg

leaf material of diploid *M. annua* of previously estimated DNA content (plants sampled from population 500a, Castelldefels sud, in which 4C = 2.86 pg ± 0.014; Buggs, 2004; Appendix 9.1) were chopped with a razor blade in 1 mL ice cold 'LB01' lysis buffer and staining solution. Buffer and staining solution were modified from Dolezel *et al*. (1989) as follows: 15 mM Tris base; 2 mM Na_2EDTA; 0.5 mM spermine tetrahydrochloride; 80 mM KCl; 20 mM NaCl; 0.1% (v/v) Triton X-100; 15mM ß-mercaptoethanol; 50 µg/mL propidium iodide; and 50 µg/mL RNAse. The subsequent suspension was filtered through 30 µm mesh 'CellTric' disposable filter (Partec GmbH, Münster, Germany) and analysed using a Becton Dickinson FACScan flow cytometer. For each sample, 10000 events were recorded in each of three runs and mean peak values were analysed using CellQuest software (Becton Dickinson). For each run, DNA content was calculated with reference to the peak of the diploid *M. annua* sample of known DNA content (in the event of a single peak being produced, it was assumed that the samples were also of diploid ploidy level). These values were thus used to infer the ploidy of samples and hence assess hybridisation success.

Following the testing of a number of individuals per cross for hybridisation success by flow cytometry, it was found that hybrids could also be reliably identified by visual inspection. Female and hermaphroditic hybrids had very low female fertility (see section 5.3.3), with fruits unfertilized and remaining small, with long feathery stigmas. In comparison, non-hybrid progeny had significantly greater female fertility and fruits developed normally (see Appendix 9.2 for photographs of parent and hybrid morphology). These differences in fruit morphology were highly apparent and in those individuals tested for hybridisation success by flow cytometry

there was a 100% association between fruit morphology and hybridisation success, hence visual identification of hybrid progeny was deemed a reliable method.

Once hybridisation success had been established and hybrid progeny identified, all hybrids were scored for sex to obtain a sex ratio for each cross. Pollen viability, female fertility, reproductive effort and sex allocation were also estimated in a selection of hybrids of each gender, grown under controlled conditions, from a range of the crosses (described in section 5.2.2). Further crosses using hybrid progeny were also subsequently undertaken in summer 2011 in order to observe the segregation of gender through a second generation. Specifically, progeny from each of the nine crosses were inbred using the same method of open-pollination as outlined above. Seeds from these crosses were likewise collected and grown to obtain sex ratios for the F_2 generation.

All hybrid sex ratios (number of males/total number of individuals) were tested for significant deviations from a 1:1 male to female sex ratio, or from a 1:1 male to hermaphrodite ratio in some cases, using goodness-of-fit tests (*G*-tests; Sokal & Rohlf, 1995).

5.2.2 Comparative morphological analysis

Seeds collected from artificial crosses described in section 5.2.1 were also used in a comparative morphological analysis of annual mercury species and their hybrids. Individuals from all parent populations used to generate hybrids were included in the analysis, in addition to seeds obtained from the following crosses (written as maternal x paternal lineage): diploid *M. annua* x *M. huetii*; *M. huetii* x diploid *M.*

annua; *M. huetii* x tetraploid *M. annua*; diploid *M. annua* x tetraploid *M. annua*; diploid *M. annua* x hexaploid (monoecious) *M. annua*; diploid *M. annua* x hexaploid (male) *M. annua*. Seeds from crosses involving tetraploid or hexaploid *M. annua* as the maternal parent were not used in this study since hybridisation success in these crosses was very low and insufficient hybrid seed was collected.

In summer 2011, approximately 100 seeds from each parent population and artificial cross were sown in soil-based compost in separate seed trays and grown in a climate-controlled glasshouse with 16 hours daylight at 22 ºC. Immediately following germination, 30 seedlings per tray were selected at random and transplanted into individual 7 x 7 cm pots of soil-based compost. Pots were arranged randomly on a glasshouse bench and watered for a period of eight weeks. Pots were spaced a minimum of 12 cm apart to avoid interference between plants and were re-randomised every seven days. After four weeks' growth, when plants could be reliably sexed, the number of individuals was reduced to a maximum of 12 per gender from each parent lineage or artificial cross. In total, 179 plants were grown.

After eight weeks' growth, all plants were harvested and the following morphological characters were measured for each individual (indices refer to leaf nodes on the main stem, with node zero denoting the cotyledons): plant height; length of each of the first five internodes; diameter of internode 1; length of petiole 3; length of leaf 3; width of leaf 3; length of branches 0 to 3; and length of the first two internodes for branches 0 and 1. Total above-ground dry vegetative biomass and the dried biomass of all male and female flower parts were also separately

measured for each individual in order to estimate reproductive effort and sex allocation as follows:

$$\text{Total Reproductive Effort (TRE)} = \frac{\text{male flower mass + female flower mass}}{\text{total above-ground biomass}}$$

$$\text{Male Reproductive Effort (MRE)} = \frac{\text{male flower mass}}{\text{total above-ground biomass}}$$

$$\text{Female Reproductive Effort (FRE)} = \frac{\text{female flower mass}}{\text{total above-ground biomass}}$$

$$\text{Sex allocation} = \frac{\text{male flower mass}}{\text{male flower mass + female flower mass}}$$

Pollen viability and female fertility were also estimated in all individuals as appropriate. Pollen viability was estimated by staining with lactophenol blue and scoring a sample of 100 pollen grains per plant (Stone *et al*., 1995), whilst female fertility was estimated by counting the proportion of female flowers per plant that were visibly setting seed.

Analysis of morphological data was performed using linear discriminant function analyses to examine whether the morphological characters measured can be used to identify hybrids from their parent lineages. Six separate analyses were conducted: the first incorporated individuals solely from the four parent populations (to ascertain whether annual mercury lineages can be distinguished from one another reliably using this method); one analysis was then conducted for each of the four hybrid types (each of which also included individuals from the relevant parent populations); and finally an analysis incorporating all individuals sampled was

performed. All of the vegetative characters measured above were used in each analysis, with the relevant hybrids and parent lineages specified as separate groups as appropriate. Two-sample *t*-tests were used to assess specific morphological differences between groups.

Differences in reproductive effort and sex allocation between hybrids and their parent lineages were assessed by analysis of variance (ANOVA). As measures of reproductive effort and sex allocation were highly bimodal in distribution (due to both unisexual and functionally hermaphroditic individuals being sampled), estimates of reproductive effort and sex allocation were analysed separately in unisexual and functionally hermaphroditic plants. Specifically, three separate ANOVAs were performed to assess differences in reproductive effort and sex allocation between: i) diploid *M. annua*, *M. huetii* and hybrids from reciprocal crosses of the two; ii) male diploid *M. annua*, male hexaploid *M. annua* and male hybrids from crosses between diploid female *M. annua* and hexaploid male *M. annua*; and iii) tetraploid *M. annua*, hermaphroditic hexaploid *M. annua* and all hermaphroditic hybrids from crosses between lineages of annual mercuries grown for this analysis (as hybrids of female *M. huetii* and tetraploid *M. annua* were found to have a reproductive effort and sex allocation significantly different to all other hermaphroditic hybrids, this group of hybrids were also analysed separately from other hermaphrodites in some cases; see section 5.3.2 for further details). In each case, ANOVAs were used to assess differences in mean estimates of reproductive effort and sex allocation between different groups of hybrids and parent lineages (a group is defined as sharing the same maternal and paternal parents; factor = GROUP). Furthermore, in the analysis of diploid *M. annua*, *M. huetii* and hybrids of

the two, differences in reproductive effort between males and females (factor = SEX), and whether the reproductive effort of different hybrid and parent groups also differed between sexes (i.e. whether there was a GROUP x SEX interaction), were also assessed, whilst in the analysis of hermaphroditic plants, differences were also assessed more broadly between all hybrid plants and all plants from parent lineages (i.e. a comparison between the two groups 'hybrids' and 'parents'; factor = HYBRID). In each ANOVA, one of total reproductive effort (TRE), male reproductive effort (MRE), female reproductive effort (FRE) or sex allocation was set as the response variable, with GROUP, SEX and/or HYBRID fitted as fixed factors. Response variables were square-root transformed where necessary in order to satisfy the assumptions of normality and homogeneity of variance.

As the fertility data did not meet the assumptions of ANOVA, Kruskal-Wallis tests were used to assess differences in mean pollen viability and female fertility between hybrids and parent lineages. Similar comparisons were made between different hybrid groups and parent lineages, as well as more broadly between all hybrid plants and all plants from parent lineages, as outlined above for the reproductive effort and sex allocation data. All analyses were conducted in *R*, version 2.8.0 (http://www.r-project.org).

5.2.3 In vivo induction of polyploidy

Seeds of diploid, dioecious *M. annua* for this experiment were bulk collected from > 100 female individuals of a single population located in Castelldefels sud, Spain (Appendix 9.1). Seeds of *M. huetii* x tetraploid *M. annua* hybrids were obtained

from the controlled reciprocal crosses between these lineages described in section 5.2.1.

To achieve artificial polyploidisation, two different methods were utilised depending on the seeds being treated. For diploid seeds and those seeds obtained from hybridisations between female *M. huetii* and tetraploid *M. annua*, approximately 40 seeds were germinated on filter paper mounted on cotton wool in each of 120 Petri dishes (90 x 15 mm) for each seed type. The cotton wool of each Petri dish was soaked in 10 mL 0.1% colchicine solution and all Petri dishes were kept on the lab bench for a period of four days to allow germination. Seedlings were then transplanted into soil-based compost in seed trays and grown under standard glasshouse conditions for six weeks. Alternatively, for seeds obtained from hybridisations between tetraploid *M. annua* and male *M. huetii*, seeds were initially sown in soil-based compost in seed trays and grown under standard glasshouse conditions. When plants were large enough, hybrid and non-hybrid plants were identified by either flow cytometry or visual inspection of fruits (see section 5.2.1) and all non-hybrids were removed. All hybrids were then treated with colchicine by taking several cuttings of lateral branches from each plant and transferring them to Petri dishes containing cotton wool soaked in 10 mL 0.1% colchicine solution. Cuttings were left in Petri dishes for 24 hours before being dipped in 10% Synergol rooting hormone (Certis) and transplanted into soil-based compost in seed trays and grown under standard glasshouse conditions. In total, approximately 200 cuttings taken from 60 hybrids were treated using this method. This alternative methodology was employed as a result of the low hybridisation success (21.7%; Table 5.1) of the tetraploid *M. annua* x male *M. huetii* cross. Hybrids thus needed to be identified

from non-hybrids before colchicine treatment in order to ensure that polyploidisation of the desired genotypes was undertaken. In contrast, hybridisation success of the female *M. huetii* x tetraploid *M. annua* cross was much greater (98.5%) and thus the genotypes of seeds could be assumed more reliably. For this reason, the more efficient method for inducing polyploidy of germinating seeds in colchicine solution was employed for these seeds.

All surviving plants from both treatments were transplanted into individual 15 cm diameter pots of soil-based compost and assessed for polyploidisation success by flow cytometry, following the procedure outlined in section 5.2.1. All plants identified as polyploids were scored for sex and were subsequently used in crosses. Specifically, all neo-tetraploids produced from diploid seed were crossed amongst one another in an isolated glasshouse, whilst three neo-hexaploids produced from female *M. huetii* x tetraploid *M. annua* hybrid seed were crossed together in a second glasshouse, using the same method of open-pollination as described in section 5.2.1. Seeds obtained from these crosses were collected and sown in seed trays to obtain sex ratios for the F_1 polyploid generation. Sex ratios (number of males/total number of individuals) of neo-polyploids were tested for significant deviations from a 1:1 sex ratio using *G*-tests (Sokal & Rohlf, 1995).

5.3 RESULTS

5.3.1 Hybrid sex ratios

Crosses between diploid *M. annua* and *M. huetii* yielded male and female hybrid progeny, irrespective of which lineage was used as the maternal and paternal parent in the hybridisation (Table 5.1; Figure 5.1). The direction of the cross did influence the sex ratio produced, however, as when diploid *M. annua* was used as the maternal parent the sex ratio was significantly female-biased (sex ratio = 0.424, $G_1 = 10.4$, $p = 0.001$), whereas when *M. huetii* acted as the maternal parent the sex ratio did not deviate significantly from 1:1 (sex ratio = 0.540, $G_1 = 2.65$, $p = 0.103$). F_2 progeny from these crosses likewise yielded males and females in ratios not significantly different from 1:1.

All hybrid progeny obtained from the female *M. huetii* x tetraploid *M. annua* cross were morphologically hermaphroditic (i.e. hybrids produced both male and female flowers, but their exceptionally low fertility, see section 5.3.3, means they cannot be regarded as functionally hermaphroditic; henceforth all references to hybrid gender will refer to morphological, rather than functional, gender). In contrast, hybrids obtained from the reciprocal cross (when *M. huetii* was used as the male parent) were either male or hermaphroditic, in a ratio not significantly different from 1:1 (sex ratio = 0.533, $G_1 = 0.80$, $p = 0.371$). Hybrid progeny from crosses between diploid *M. annua* and tetraploid *M. annua* showed a similar pattern; when tetraploid *M. annua* was used as the paternal lineage, all hybrid progeny were hermaphroditic, whereas when diploid *M. annua* was used as the male parent, hybrids were either male or hermaphroditic, again with no significant sex ratio deviation from 1:1 (sex ratio = 0.455, $G_1 = 0.09$, $p = 0.763$). Crosses between diploid *M. annua* and

hexaploid *M. annua* also produced a similar pattern in the sex ratio of hybrids. When diploid *M. annua* females were crossed with hexaploid *M. annua* hermaphrodites, all hybrid progeny were hermaphroditic. However, when diploid *M. annua* females were crossed with hexaploid *M. annua* male plants, male progeny were produced alongside hermaphrodites, again with a sex ratio not significantly different from 1:1 (sex ratio = 0.533, $G_1 = 0.33$, $p = 0.564$). Finally, when diploid *M. annua* was used as the male parent, hybrids were either male or hermaphroditic, with no significant deviation in sex ratio from 1:1 (sex ratio = 0.357, $G_1 = 1.16$, $p = 0.282$).

With the exception of hybrids of diploid *M. annua* and *M. huetii*, further crosses using hybrids failed to produce any viable seeds. Hermaphrodites obtained from *M. huetii* x tetraploid *M. annua*, tetraploid *M. annua* x *M. huetii*, tetraploid *M. annua* x diploid *M. annua* and hexaploid *M. annua* x diploid *M. annua* crosses set zero seed, whilst hermaphrodites obtained from diploid *M. annua* x tetraploid *M. annua*, diploid x hexaploid male *M. annua* and diploid *M. annua* x hexaploid hermaphroditic *M. annua* hybridisations produced just a single seed per cross, all of which failed to germinate. The sex expression of F_2 progeny from these crosses could therefore not be examined (see section 5.3.3 for a full analysis of hybrid fertility).

5.3.2 Reproductive effort and sex allocation

Analyses of total reproductive effort in diploid *M. annua*, *M. huetii* and hybrids from reciprocal crosses of the two revealed a highly significant GROUP x SEX interaction, indicating that the reproductive effort of different hybrid and parent

groups also differed between sexes ($F_{3, 80} = 14.5$, $p < 0.001$; Table 5.2; Figure 5.2). Specifically, total reproductive effort was significantly greater in male hybrids generated from crosses between female diploid *M. annua* and male *M. huetii* than in all other plants, including males obtained from the reciprocal cross, diploid *M. annua* males and *M. huetii* males. In contrast, reproductive effort was significantly lower in all female hybrids than in male hybrids and diploid *M. annua* females, regardless of the direction of the hybridisation. Total allocation of resources to reproduction was also lower in male and female *M. huetii* than in diploid *M. annua* and male hybrids of the two. In male hybrids produced from crosses between female diploid *M. annua* and male hexaploid *M. annua*, total reproductive effort was significantly greater than in either diploid or hexaploid males ($F_{2, 25} = 45.8$, $p < 0.001$; Table 5.3; Figure 5.3).

In all hermaphroditic plants for which reproductive effort and sex allocation were assessed, total reproductive effort was significantly lower in hybrids compared to individuals from tetraploid and hexaploid *M. annua* parent populations ($F_{1, 73} = 50.5$, $p < 0.001$; Table 5.4; Figure 5.4). This difference was largely attributable to a significant reduction in male reproductive effort in hybrids compared to parents ($F_{1, 73} = 74.8$, $p < 0.001$; Figure 5.4). Female reproductive effort was significantly reduced in *M. huetii* x tetraploid *M. annua* hybrids relative to its tetraploid *M. annua* progenitor ($F_{1, 22} = 74.1$, $p < 0.001$), but there were no significant differences in the allocation of resources to female function between all other hybrids and hermaphrodites from parent populations ($F_{1, 61} = 1.72$, $p = 0.194$). Sex allocation was subsequently significantly more female-biased in hermaphroditic hybrids relative to individuals from parent populations ($F_{1, 61} = 82.4$, $p < 0.001$), except in

the case of *M. huetii* x tetraploid *M. annua* hybrids where sex allocation was quantitatively more male-biased relative to tetraploid *M. annua* ($F_{1, 22} = 46.7$, $p < 0.001$).

It is worth noting that estimates of reproductive effort and sex allocation in hybrids do not appear to have been influenced by their high sterility levels (see section 5.3.3). All estimates of reproductive effort and sex allocation were made using the dried biomass of all male and female flower parts which, visually at least, did not appear to be influenced by sterility, with male and female flowers produced in hybrids similar to those of parent lineages. (See section 5.4.2 for further discussion of this point.)

5.3.3 Hybrid fertility

Pollen viability was significantly lower in hybrids than individuals from parent populations ($H_1 = 48.1$, $p < 0.001$; Figure 5.5). Viability did not differ significantly, however, between progeny from different hybrid crosses ($H_5 = 2.24$, $p = 0.815$). Female fertility was likewise significantly lower in hybrids compared to individuals from parent populations ($H_1 = 85.7$, $p < 0.001$; Figure 5.6). The proportion of female flowers setting seed was significantly greater in hybrid progeny obtained from crosses between diploid *M. annua* and *M. huetii* (in both directions) than in other hybrid progeny ($H_1 = 23.7$, $p < 0.001$), with female fertility not differing significantly between hybrid progeny from all other crosses assessed ($H_3 = 3.04$, $p = 0.385$).

5.3.4 Comparative morphological analysis

Discriminant function analysis of individuals from parent populations correctly classified all *M. huetii*, diploid *M. annua* and hexaploid *M. annua* individuals (Figure 5.7). Individuals of *M. huetii* were smaller than individuals of *M. annua* lineages, with smaller leaves and narrower main stems ($p < 0.001$ for each), findings in correspondence with previous observations and analyses (Durand & Durand, 1985; Obbard *et al*., 2006a). Stem width was the best discriminator between diploid and polyploid *M. annua*; diploid *M. annua* had wider main stems than tetraploid and hexaploid lineages ($p = 0.002$). Tetraploid *M. annua* individuals were harder to distinguish from hexaploid *M. annua*, with 16.7% of tetraploids misclassified as hexaploids. These results are in correspondence with those of Obbard *et al*. (2006a), who likewise found it difficult to accurately distinguish morphologically between tetraploid and hexaploid *M. annua* lineages.

Discriminant function analysis of *M. huetii*, diploid *M. annua* and hybrids of the two correctly assigned all *M. huetii* and diploid *M. annua* individuals, but was unable to distinguish reliably between hybrids, with 12.8% misclassified as hybrids from the reciprocal cross (Figure 5.8a). Hybrids were of intermediate height, vegetative mass, leaf size and stem width relative to their progenitors ($p < 0.025$ for each; see Appendix 9.2 for photographs of parent and hybrid morphology). Analysis of *M. huetii*, tetraploid *M. annua* and hybrids of the two likewise correctly assigned all *M. huetii* individuals, but misallocated one tetraploid as a hybrid and one hybrid as a tetraploid (Figure 5.8b). In contrast, analysis of diploid and tetraploid *M. annua* and their hybrid correctly classified all individuals, with hybrids of significantly greater vegetative mass and possessing larger leaves relative to their parent lineages ($p < 0.01$ for each; Figure 5.8c). Analysis of diploid and hexaploid *M. annua* and hybrids

of the two correctly assigned all diploid individuals, but was unable to distinguish reliably between hexaploids and hybrids, with 14.3% of hexaploid and hybrid individuals misclassified (Figure 5.8d).

A discriminant function analysis of all plants sampled misclassified 21.2% of individuals (although all *M. huetii*, diploid *M. annua* and diploid *M. annua* x tetraploid *M. annua* hybrids were correctly assigned), reflecting the high morphological similarity of hybrids and parent lineages.

5.3.5 Neo-polyploids

Fourteen neo-tetraploids were produced by in vivo polyploidisation of diploid *M. annua* seed, all of which were confirmed to be of tetraploid ploidy level by flow cytometry (Table 5.5). Of these fourteen plants, nine were identified as female and five as male, giving a sex ratio not significantly different from 1:1 (sex ratio = 0.357, $G_1 = 1.16$, $p = 0.282$). Fertility of the nine females produced was very low and crosses amongst neo-tetraploids yielded just 17 seeds, of which eight germinated, giving rise to six male and two female plants.

Ten neo-hexaploids were produced by in vivo polyploidisation of triploid hybrid seed obtained from female *M. huetii* x tetraploid *M. annua* crosses. All ten plants were scored as monoecious hermaphrodites. Open pollination crosses between three of these neo-hexaploids produced a large amount of viable seed (>100 seeds per plant), which gave rise to exclusively monoecious progeny. In vivo polyploidisation of tetraploid *M. annua* x male *M. huetii* hybrids failed to produce any neo-

hexaploids, however, as no cuttings from hybrid plants survived colchicine treatment and transplantation.

Table 5.1 The morphological gender, sex ratios (number of males/total number of individuals) and fertility of progeny produced from crosses between various lineages of the annual mercury clade. Hybridisation success (the percentage of hybrids out of total progeny produced) is also displayed for each cross. * Denotes values of *G* significant at $p < 0.05$. Pollen viability represented as the mean proportion of pollen grains stained with lactophenol blue (± one standard error) per cross. Female fertility measured as the mean proportion of female flowers visibly setting seed (± one standard error) per cross.

Maternal lineage	Paternal lineage	Males	Females	Hermaphrodites	Sex ratio	*G*	Pollen viability	Female fertility	Hybridisation success
M. annua (2*x*)	*M. huetii*	190	258	0	0.424	10.4*	0.195 ± 0.072	0.061 ± 0.019	99.3
M. huetii	*M. annua* (2*x*)	222	189	0	0.540	2.65	0.186 ± 0.081	0.034 ± 0.012	99.3
M. annua (4*x*)	*M. huetii*	96	0	84	0.533	0.80	-	-	21.7
M. huetii	*M. annua* (4*x*)	0	0	521	0.000	-	0.186 ± 0.068	0.001 ± 0.001	98.5
M. annua (2*x*)	*M. annua* (4*x*)	0	0	88	0.000	-	0.206 ± 0.050	0.005 ± 0.003	77.2
M. annua (4*x*)	*M. annua* (2*x*)	5	0	6	0.455	0.09	-	-	16.2
M. annua (2*x*)	*M. annua* (6*x*), male	40	0	35	0.533	0.33	0.161 ± 0.050	0.006 ± 0.005	94.9
M. annua (2*x*)	*M. annua* (6*x*), hermaphrodite	0	0	31	0.000	-	0.132 ± 0.051	0.001 ± 0.001	81.6
M. annua (6*x*)	*M. annua* (2*x*)	5	0	9	0.357	1.16	-	-	46.7

Table 5.2 Results of the analysis of variance (ANOVA) assessing differences in total reproductive effort between diploid *M. annua*, *M. huetii* and hybrids from reciprocal crosses of the two. Values significant at $p < 0.05$ are shown in bold.

	d.f.	**Adjusted Sum of Squares**	**Mean Square**	***F***	***p***
Sex	1	0.3390	0.3390	91.5	**< 0.001**
Group	3	0.3908	0.1303	35.2	**< 0.001**
Sex x Group	3	0.1612	0.0537	14.5	**< 0.001**
Error	80	0.2963	0.0037		

Table 5.3 Results of the analysis of variance (ANOVA) assessing differences in total reproductive effort between male diploid *M. annua*, male hexaploid *M. annua* and male hybrids from crosses between diploid female *M. annua* and hexaploid male *M. annua*. Values significant at $p < 0.05$ are shown in bold.

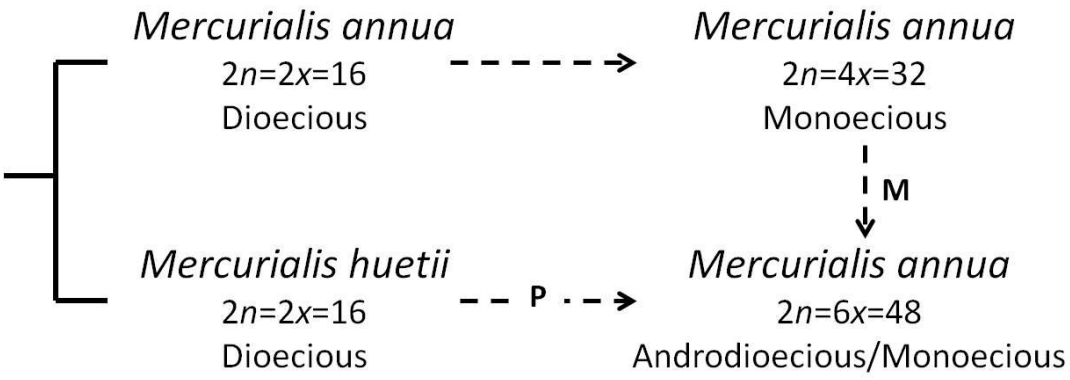
	d.f.	**Adjusted Sum of Squares**	**Mean Square**	***F***	***p***
Group	2	0.1735	0.0868	45.8	**< 0.001**
Error	25	0.0473	0.0019		

Table 5.4 Results of the analysis of variance (ANOVA) assessing differences in reproductive effort and sex allocation between tetraploid *M. annua*, hermaphroditic hexaploid *M. annua* and hermaphroditic hybrids from crosses between various lineages of annual mercuries. TRE = total reproductive effort; MRE = male reproductive effort; FRE = female reproductive effort. Values significant at $p < 0.05$ are shown in bold.

		TRE				**MRE**				**FRE**				**Sex allocation**			
	d.f.	**Adjusted Sum of Squares**	**Mean Square**	***F***	***p***	**Adjusted Sum of Squares**	**Mean Square**	***F***	***p***	**Adjusted Sum of Squares**	**Mean Square**	***F***	***p***	**Adjusted Sum of Squares**	**Mean Square**	***F***	***p***
Group	4	0.1656	0.0414	23.2	**< 0.001**	0.4236	0.1059	31.8	**< 0.001**	0.3599	0.0900	17.8	**< 0.001**	4.0826	1.0206	46.7	**< 0.001**
Error	70	0.1251	0.0018			0.2331	0.0033			0.3546	0.0051			1.5308	0.0219		
Hybrid	1	0.1188	0.1188	50.5	**< 0.001**	0.3323	0.3323	74.8	**< 0.001**	0.0851	0.0851	9.87	**0.002**	0.8195	0.8195	12.5	**0.001**
Error	73	0.1719	0.0024			0.3243	0.0044			0.6294	0.0086			4.7939	0.0657		

Table 5.5 Summary of the morphological gender and DNA content (C-value) of the neo-polyploids produced by colchicine treatment in this study. The DNA content of each individual was calculated with reference to diploid *M. annua* of previously estimated DNA content. Estimates of DNA content were then used to infer ploidy by comparison with the DNA content of parent material (diploid *M. annua* 4C = 2.86 pg ± 0.014; tetraploid *M. annua* 4C = 5.23 pg ± 0.009; *M. huetii* 4C = 2.85 pg ± 0.007; Buggs, 2004).

Plant ID	Parent material	Parent ploidy	Gender	4C DNA (pg)	Standard error	Inferred ploidy
2.2	Wild *M. annua*	2*x*	Female	5.52	0.034	4*x*
2.3	Wild *M. annua*	2*x*	Female	5.71	0.047	4*x*
2.4	Wild *M. annua*	2*x*	Female	5.83	0.029	4*x*
2.6	Wild *M. annua*	2*x*	Female	5.61	0.041	4*x*
2.9	Wild *M. annua*	2*x*	Male	5.56	0.014	4*x*
2.10	Wild *M. annua*	2*x*	Male	5.72	0.027	4*x*
2.11	Wild *M. annua*	2*x*	Female	5.88	0.031	4*x*
2.14	Wild *M. annua*	2*x*	Male	5.74	0.030	4*x*
2.15	Wild *M. annua*	2*x*	Female	5.62	0.040	4*x*
2.16	Wild *M. annua*	2*x*	Male	5.94	0.019	4*x*
2.18	Wild *M. annua*	2*x*	Female	5.67	0.012	4*x*
2.19	Wild *M. annua*	2*x*	Female	5.75	0.028	4*x*
2.20	Wild *M. annua*	2*x*	Male	5.71	0.056	4*x*
2.25	Wild *M. annua*	2*x*	Female	5.59	0.031	4*x*
3.2	*M. huetii* x *M. annua* (4*x*) hybrid	3*x*	Hermaphrodite	8.01	0.047	6*x*
3.3	*M. huetii* x *M. annua* (4*x*) hybrid	3*x*	Hermaphrodite	8.15	0.041	6*x*
3.5	*M. huetii* x *M. annua* (4*x*) hybrid	3*x*	Hermaphrodite	7.88	0.058	6*x*
3.7	*M. huetii* x *M. annua* (4*x*) hybrid	3*x*	Hermaphrodite	8.14	0.032	6*x*
3.8	*M. huetii* x *M. annua* (4*x*) hybrid	3*x*	Hermaphrodite	8.17	0.030	6*x*
3.9	*M. huetii* x *M. annua* (4*x*) hybrid	3*x*	Hermaphrodite	7.93	0.045	6*x*
3.10	*M. huetii* x *M. annua* (4*x*) hybrid	3*x*	Hermaphrodite	8.26	0.060	6*x*
3.11	*M. huetii* x *M. annua* (4*x*) hybrid	3*x*	Hermaphrodite	8.17	0.037	6*x*
3.12	*M. huetii* x *M. annua* (4*x*) hybrid	3*x*	Hermaphrodite	8.09	0.021	6*x*
3.20	*M. huetii* x *M. annua* (4*x*) hybrid	3*x*	Hermaphrodite	7.91	0.058	6*x*



Crosses between annual mercury lineages performed in this study:

Maternal parent		Paternal parent		Hybrid sex ratio
M. annua (2*x*), female	x	*M. huetii*, male	→	3:4 male:female
M. huetii, female	x	*M. annua* (2*x*), male	→	1:1 male:female
M. annua (4*x*), hermaphrodite	x	*M. huetii*, male	→	1:1 male:hermaphrodite
M. huetii, female	x	*M. annua* (4*x*), hermaphrodite	→	100% hermaphrodite
M. annua (2*x*), female	x	*M. annua* (4*x*), hermaphrodite	→	100% hermaphrodite
M. annua (4*x*), hermaphrodite	x	*M. annua* (2*x*), male	→	1:1 male:hermaphrodite
M. annua (2*x*), female	x	*M. annua* (6*x*), male	→	1:1 male:hermaphrodite
M. annua (2*x*), female	x	*M. annua* (6*x*), hermaphrodite	→	100% hermaphrodite
M. annua (6*x*), hermaphrodite	x	*M. annua* (2*x*), male	→	1:1 male:hermaphrodite

Figure 5.1 Hypothesised relationships between lineages of the annual mercury clade used to generate hybrids by artificial reciprocal crosses in this study, followed by a summary of the crosses performed and the approximate sex ratio of hybrids produced from each cross. Filled lines indicate phylogenetic relationships between species and dashed arrows represent proposed hybridisation and/or polyploidisation events; M indicates proposed maternal parentage and P represents proposed paternal parentage. Figure based on Obbard *et al.* (2006b).

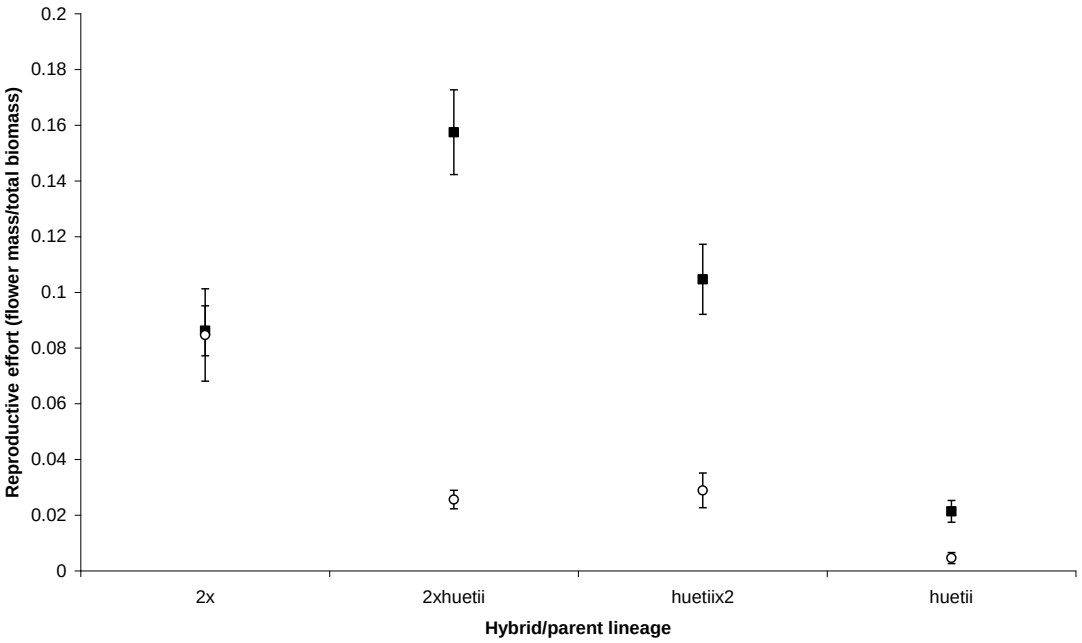


Figure 5.2 Mean (± one standard error) total reproductive effort of male (filled squares) and female (open circles) diploid *M. annua*, *M. huetii* and hybrids from reciprocal crosses of the two. Crosses written as maternal x paternal lineage, with *M. annua* lineages denoted by ploidy level, e.g. "2xhuetii" = diploid *M. annua* x *M. huetii*.

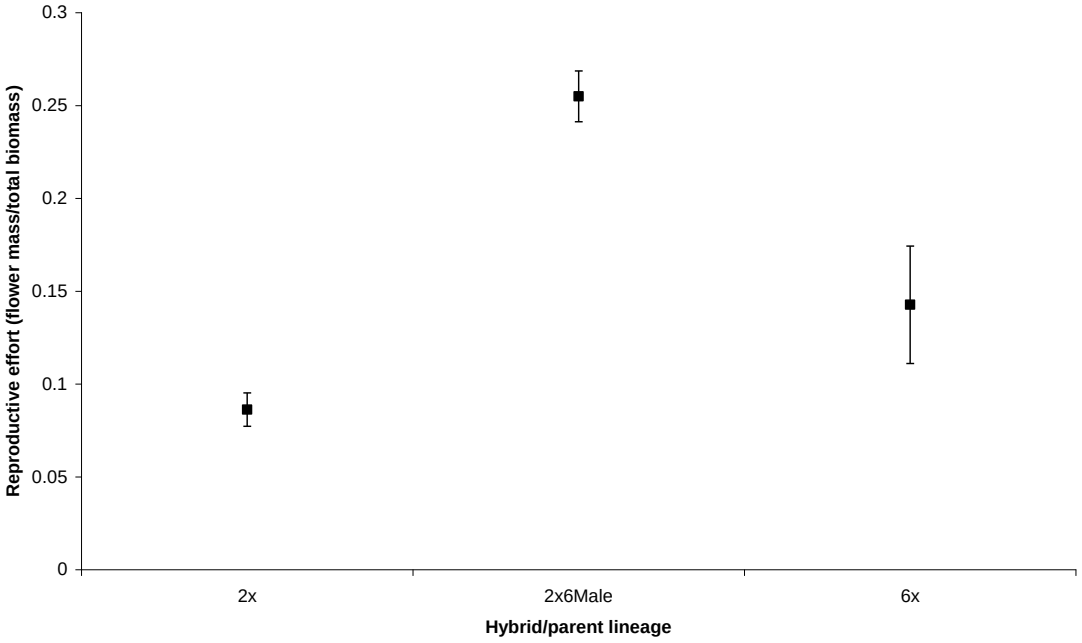


Figure 5.3 Mean (± one standard error) total reproductive effort of male diploid *M. annua*, male hexaploid *M. annua* and male hybrids from crosses between diploid female *M. annua* and hexaploid male *M. annua*. See Figure 5.2 legend for details of hybrid abbreviations.

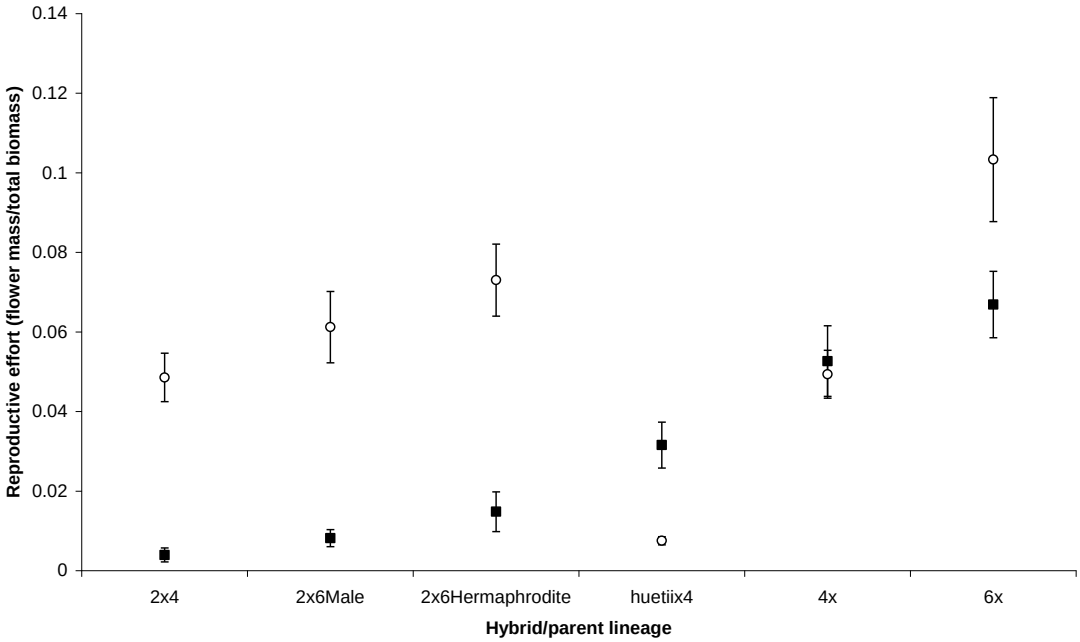


Figure 5.4 Mean (± one standard error) male (filled squares) and female (open circles) reproductive effort of tetraploid *M. annua*, hermaphroditic hexaploid *M. annua* and hermaphroditic hybrids from crosses between various lineages of annual mercuries. See Figure 5.2 legend for details of hybrid abbreviations.

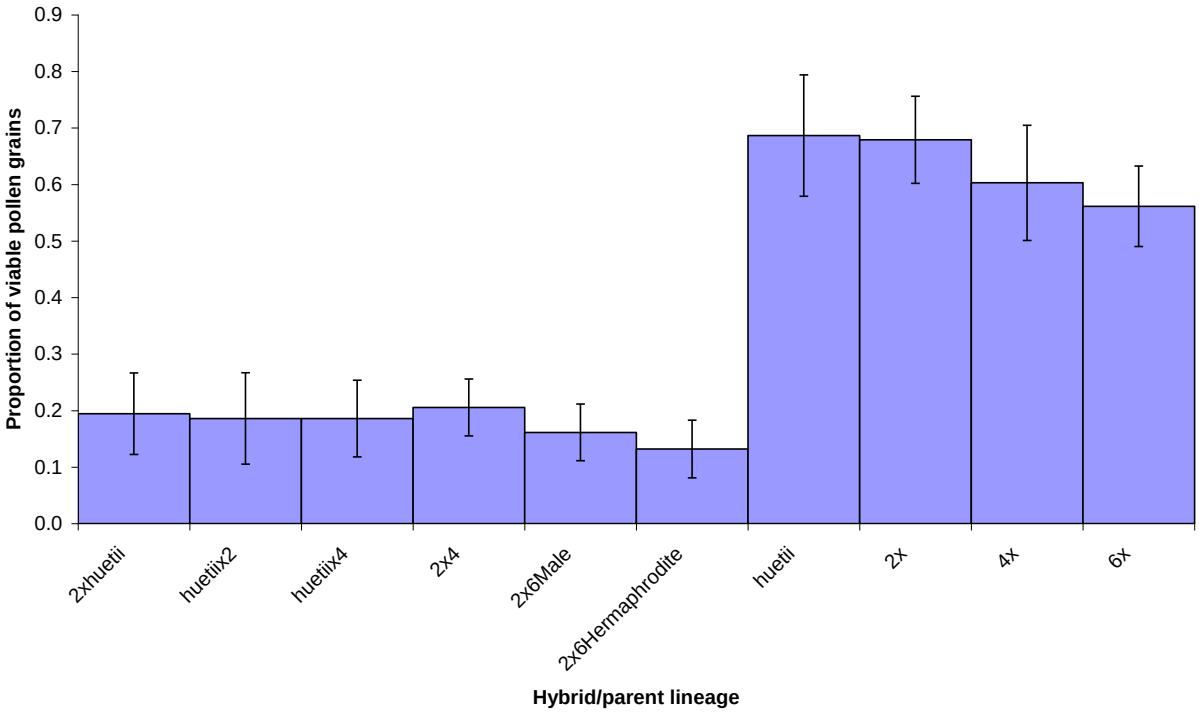


Figure 5.5 Mean (± one standard error) pollen viability of hybrids from the six cross types and four parent lineages assessed. See Figure 5.2 legend for details of hybrid abbreviations.

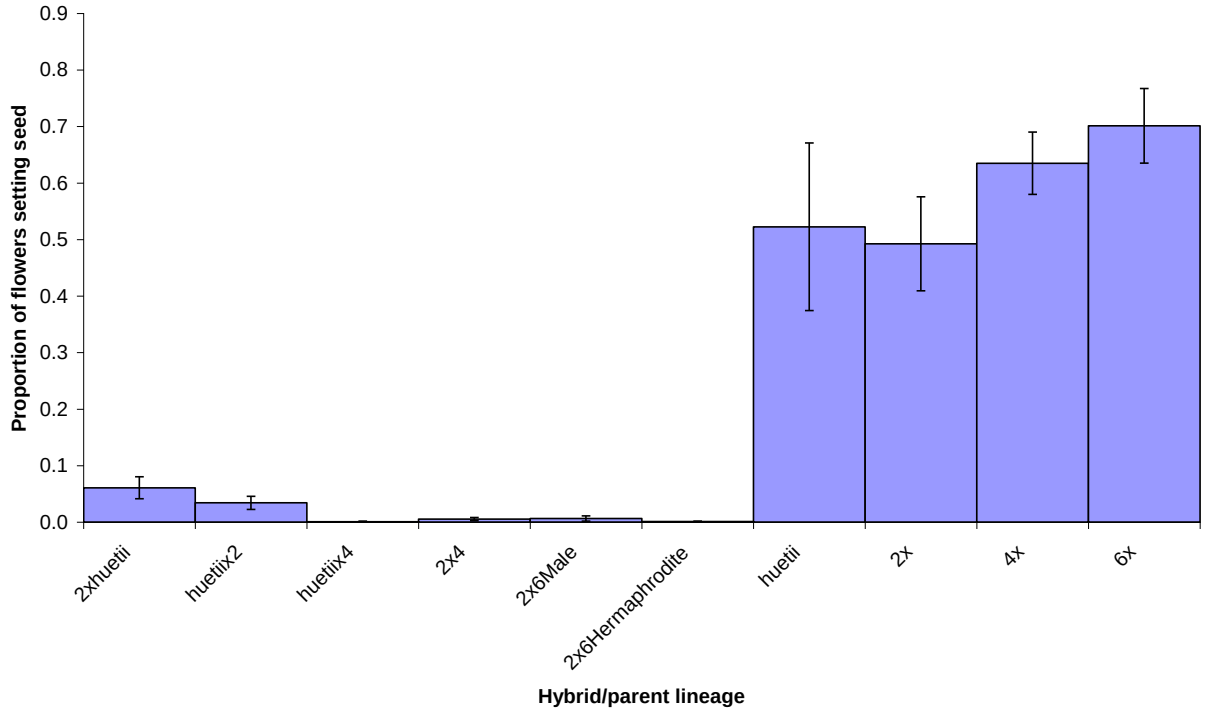


Figure 5.6 Mean (± one standard error) female fertility of hybrids from the six cross types and four parent lineages assessed. See Figure 5.2 legend for details of hybrid abbreviations.

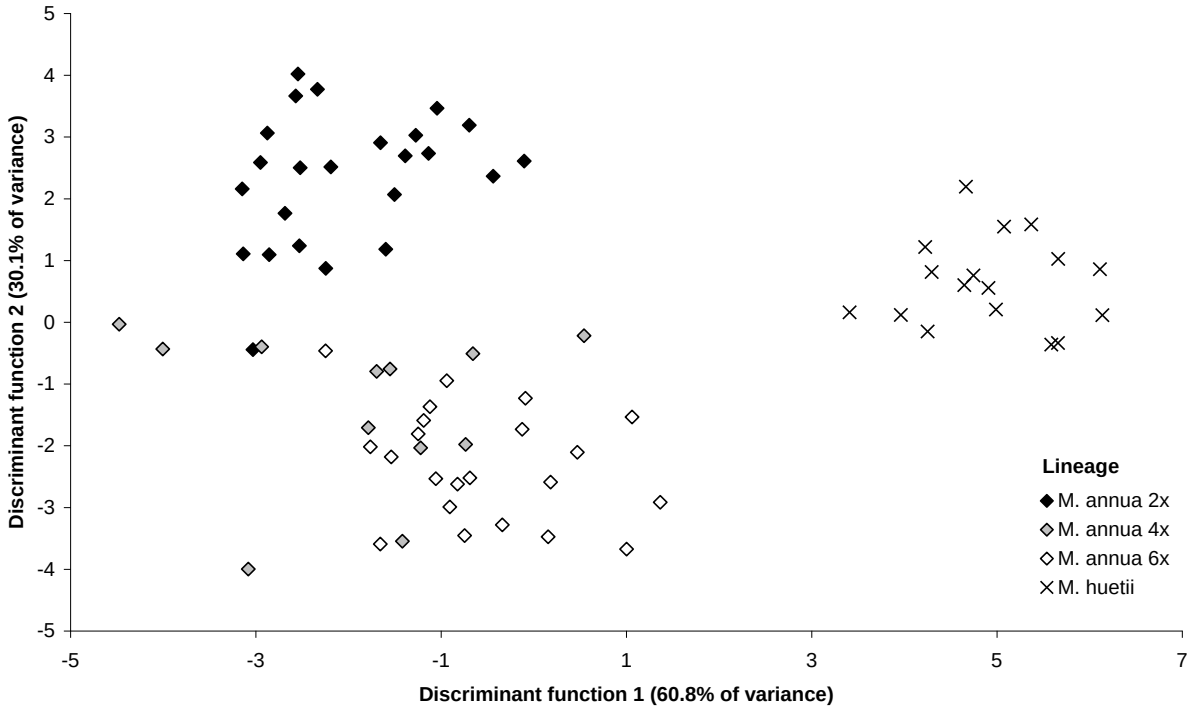


Figure 5.7 Plot of the first two discriminant functions of individuals of parent lineages grown as part of the comparative morphological analysis. All *M. huetii*, diploid *M. annua* and hexaploid *M. annua* individuals were correctly classified, whilst 16.7% of tetraploids were misclassified as hexaploids.

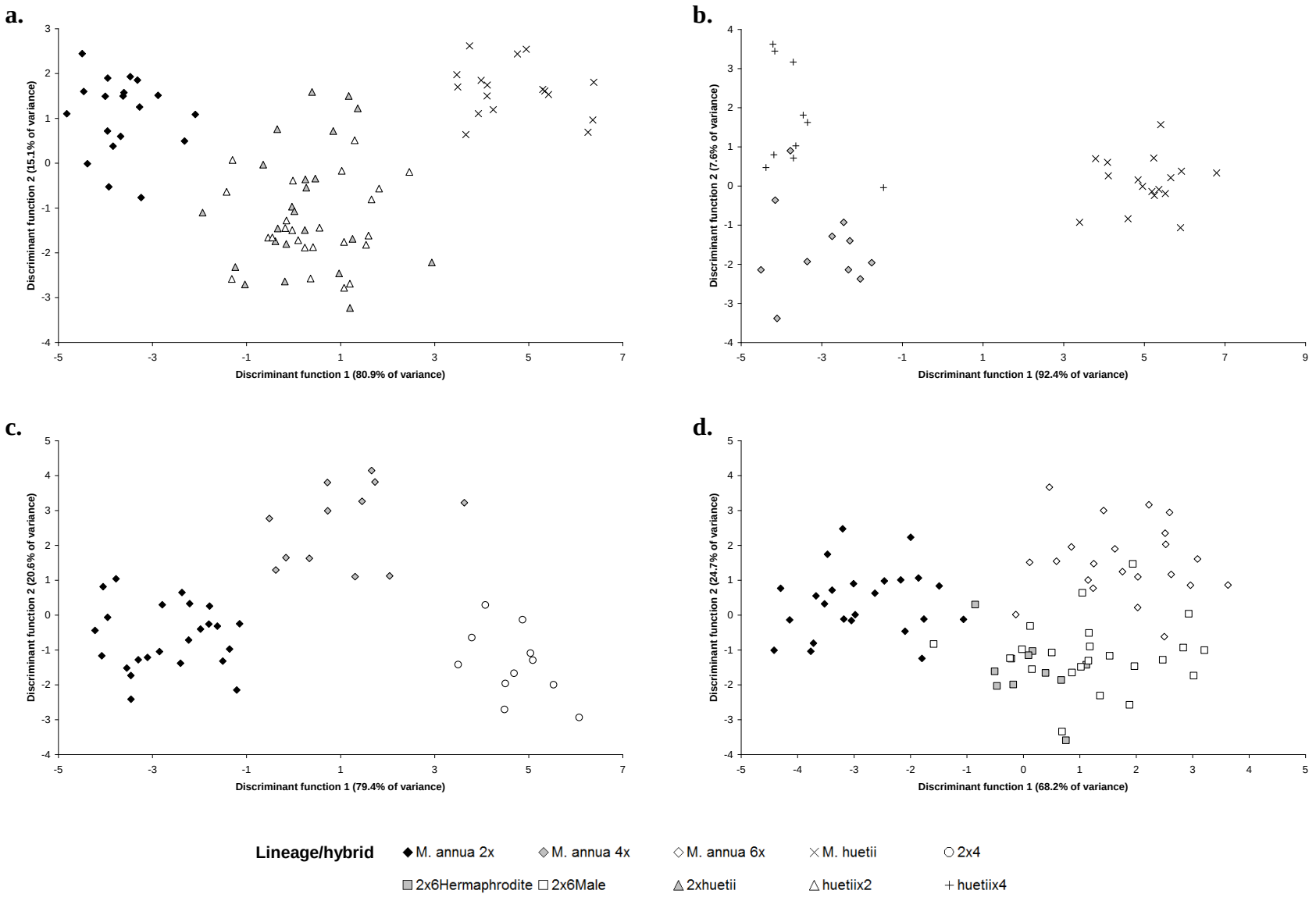


Figure 5.8 Plots of the first two discriminant functions of hybrids and associated progenitors grown as part of the comparative morphological analysis: a. *M. huetii*, diploid *M. annua* and hybrids; b. *M. huetii*, tetraploid *M. annua* and hybrid; c. diploid and tetraploid *M. annua* and hybrid; and d. diploid and hexaploid *M. annua* and hybrids.

5.4 DISCUSSION

5.4.1 Conserved sex determination across the annual mercury clade

My results point towards a conserved mechanism of single-locus genetic sex determination, with male heterogamety, across the annual mercury clade. All hybridisations involving pure male plants as the paternal parent, whether males of diploid *M. annua*, hexaploid *M. annua* or *M. huetii*, yielded male progeny, whilst no cross that did not involve a pure male plant produced male progeny. In crosses involving male plants, male progeny were produced in a ratio of 1:1 with either females or hermaphrodites (except in the case of the diploid female *M. annua* x male *M. huetii* cross, where significant departure from 1:1 was detected, discussed in section 5.4.2), indicating that the inheritance of a dominant allele at a single locus gives rise to males in each lineage, regardless of the origin of the allele. The male-determining alleles of diploid *M. annua*, hexaploid *M. annua* and *M. huetii* thus appear to be developmentally functional in all lineages of annual mercuries tested in this study (i.e. alleles were capable of producing a male phenotype of male flowers borne on erect axillary peduncles; low fertility in inter-ploidy hybrids was likely caused by meiotic irregularities), suggesting a conserved mechanism of sex determination across the annual mercury clade. Furthermore, the inheritance of just a single copy of a male-determining allele appears sufficient for the development of a male phenotype, suggesting that sex in the annual mercuries may be determined by an 'active-Y' mechanism analogous to that found in mammals. For example, consider crosses of diploid male *M. annua* with monoecious tetraploid and hexaploid *M. annua*; male progeny were produced in a ratio of 1:1 with hermaphrodites despite triploid and tetraploid hybrids presumably inheriting just a single male-determining allele from their diploid progenitor.

Although my results are consistent with the possibility of a single conserved male-determining gene across the annual mercury clade, it isn't possible to rule out the existence of different male-determining genes at different loci in each lineage, but with a conserved downstream mechanism of sex determination across the clade. For example, turnovers in sex-determining mechanisms may have occurred in some lineages, with the recruitment of a new locus controlling the assignment of gender but which utilises the same developmental pathways for regulating sexual expression as other lineages, hence the developmental functionality of a male-determining allele from one lineage in other lineages across the clade. To test for this scenario, crosses between sex-reversed plants of different lineages could be undertaken through multiple generations to enable male-determining genes from different lineages to be brought together in hybrid progeny which, in the event of lineages possessing different male-determining genes, would be predicted to yield male-biased sex ratios in F_2 progeny as male-determining alleles at multiple potentially independently segregating loci are inherited. Alternatively, assessing the homology of sex-determining genes between lineages by molecular methods would permit examination of the conservation of male-determining genes and sex-determining mechanisms across the annual mercury clade (see Chapter 3).

Whether female or hermaphroditic progeny were produced alongside males in crosses involving male plants appears to depend upon the specific lineages involved in the cross; when a polyploid lineage (i.e. tetraploid or hexaploid *M. annua*) was involved, hermaphroditic progeny were produced, whereas when only diploid lineages were involved (i.e. diploid *M. annua* or *M. huetii*), females were produced.

Moreover, all hybridisations in which monoecious plants (either tetraploid or hexaploid *M. annua*) were used as the paternal lineage yielded exclusively hermaphroditic progeny. These observations can be explained on the basis of a simple genetic model, which I outline below.

5.4.2 A model for sex determination in the annual mercuries

Males arise by the inheritance of a dominant allele at a single locus in all lineages of annual mercuries, whether in natural populations of dioecious *M. annua*, androdioecious *M. annua* and *M. huetii*, or in the hybrids discussed above. This allele leads to the production of a male phenotype consisting of staminate flowers borne on erect axillary peduncles. As discussed above, the specific gene responsible for the determination of males may be conserved across the annual mercury clade, but it is also possible that different genes may perform the same function in different lineages. Nevertheless, the downstream mechanism of sex determination is apparently conserved across the clade since male-determining alleles from dioecious *M. annua*, androdioecious *M. annua* and *M. huetii* were capable of producing a male phenotype in the genetic background of all other lineages tested in this study.

In the absence of this allele, individuals develop as either females or monoecious hermaphrodites, depending upon the inheritance of alleles controlling axillary male flower development in a female or hermaphrodite background. In dioecious *M. annua* and *M. huetii*, for example, male flower development is completely suppressed in females, presumably by male-sterility genes which theory predicts become established and genetically linked to male-determining loci during the evolution of unisexuality (see section 2.1; Charlesworth & Charlesworth, 1978a;

1980; Rice, 1984). In tetraploid and hexaploid *M. annua*, however, axillary male flower development is restored, giving rise to monoecy. Differences between monoecious hermaphrodites and females in genes controlling male flower development are potentially fixed elsewhere in the genome, not necessarily at the same locus or loci. Interestingly, when female-determining and hermaphrodite-determining genes were brought together in hybrids, for example in crosses of diploid *M. annua* or *M. huetii* with tetraploid or hexaploid *M. annua*, progeny were morphologically hermaphroditic, with no females produced. This observation suggests that genes that cause the production of axillary staminate flowers in an otherwise female background show dominant expression and are capable of restoring staminate flower production in spite of the presence of putative suppressors inherited from dioecious systems. However, although hermaphroditic hybrids produced both male and female flowers, their male reproductive effort (the dry mass of all male flower parts per unit total biomass) was significantly lower than monoecious plants from tetraploid and hexaploid parent populations. Indeed, visual inspection of hybrids revealed very few male flowers. In comparison, resource allocation to female reproductive function was not significantly different between hybrids and parents (except in the case of *M. huetii* x tetraploid *M. annua* hybrids, discussed below). These results suggest that the male-sterility effects of alleles inherited from dioecious systems do still act, to a certain extent, in hermaphroditic hybrids of dioecious and monoecious lineages and partially suppress axillary male flower development.

With regard to the male-biased sex allocation of monoecious hybrids produced from crosses between female *M. huetii* and tetraploid *M. annua*, this was the only cross in

which the sex allocation of monoecious hybrids of *M. huetii* parentage was estimated. Female *M. huetii* individuals, plus female hybrids produced from crosses between diploid *M. annua* and *M. huetii*, had a significantly lower reproductive effort than diploid *M. annua* females, suggesting that low female allocation may be characteristic of *M. huetii* and, consequently, its hybrids. The high reproductive effort of male hybrids produced from crosses of female diploid with male *M. huetii* and with male hexaploid *M. annua* is also difficult to explain in terms of the genetic model proposed above, given that all male hybrids are assumed to possess a single dominant allele conferring maleness. However, given that the coming together of divergent genomes is likely to disrupt patterns of gene expression, this explanation may best account for the differences observed in reproductive effort.

It is possible that the high sterility levels of hybrids may have influenced measures of reproductive effort and sex allocation; for example, low seed set may reduce estimates of female reproductive function, whereas male sterility may cause an excess of male flowers to be produced in an attempt to compensate for low fertility. However, all estimates of reproductive effort and sex allocation in this study were made using the dried biomass of all male and female flower parts which, visually at least, did not appear to be influenced by sterility; male and female flowers produced in hybrids were similar to those of parent lineages, with just the viability of pollen and seeds reduced. Moreover, if patterns of hybrid sex allocation were influenced by sterility levels then one might expect them to be affected uniformly across all hybrids, given all hybrids had low levels of male and female fertility. This was not the case, however, suggesting that other factors, potentially variation in gene

expression controlling flower development and sex expression, were responsible for the patterns observed.

Of all of the crosses between annual mercury lineages undertaken in this study, all except those between female diploid *M. annua* and male *M. huetii* yielded sex ratios which support the model of conserved sex determination proposed above. The significantly female-biased sex ratio obtained from this cross does not necessarily indicate a more complicated mechanism of sex determination is in operation, however. As discussed in Chapter 2, mechanisms which bias sex ratios away from those expected purely from the Mendelian segregation of sex-determining genes are well documented in dioecious plants (Werren & Beukeboom, 1998; de Jong & Klinkhamer, 2002; Barrett et *al*., 2010) and the biased sex ratios reported in full-sib and half-sib seed families of dioecious *M. annua* in this thesis suggest sex ratio distorters may be present in the species (Chapter 2). The female-biased sex ratio produced here, therefore, could be explained by such factors. Moreover, given the genetic divergence between dioecious *M. annua* and *M. huetii*, each species may possess unique sex ratio distorters, to which the other does not possess appropriate modifiers with which the effects are counteracted. I therefore suppose that the biased sex ratio obtained in this study may be attributable to such factors and that single-locus sex determination with male heterogamety is conserved across the annual mercury clade.

Similar sex ratios to those reported here have been obtained from crosses amongst monoecious and dioecious populations of *Sagittaria latifolia* (Dorken & Barrett, 2004) and *Ecballium elaterium* (Galán, 1951). In these cases, male-determining

alleles were likewise interpreted as being dominant to monoecious-determining alleles, which were in turn dominant to female-determining alleles. In contrast, crosses between androdioecious *Datisca glomerata* and its dioecious sister species *D. cannabina* yielded only male and female offspring, from which it was concluded that hermaphroditism is recessive to femaleness (Wolf *et al.*, 2001). Similarly, crosses between dioecious *Bryonia dioica* and monoecious *B. alba* also yielded exclusively unisexual hybrid progeny (Correns, 1903; 1907), whilst in *Urtica dioica* a complicated model of sex determination involving four alleles segregating at a single locus has been proposed in which dioecious-determining alleles are generally, though not completely, dominant over monoecious-determining alleles (Glawe & de Jong, 2008). These varying models of sex determination likely reflect the different evolutionary histories of the species and their different sexual systems, i.e. how monoecy and dioecy arose, and which of the mating systems is ancestral. In *D. glomerata*, for example, androdioecy has been proposed to have evolved from a formerly dioecious population in which a recessive mutation arose that enabled pollen production in females, hence explaining the recessive nature of hermaphroditism to femaleness in interspecific crosses with *D. cannabina* (Wolf *et al.*, 2001). Amongst annual mercuries, the picture is complicated by the polyploidisation and hybridisation events hypothesised to have taken place in the system, functional hermaphroditism being restricted solely to polyploid lineages (Obbard *et al.*, 2006b). Given the evolutionary history of the annual mercury clade, it is interesting, for example, to consider the function of genes in polyploid lineages at loci homologous to those which determined sex in their progenitors. I will now discuss this question alongside theories for the evolution of androdioecy and monoecy in the annual mercury clade more generally.

5.4.3 The evolution of androdioecy in hexaploid *M. annua*

Given the hypothesised evolutionary history of the annual mercury clade and the relationships between its members, it is perhaps not surprising that the dominant male-determining allele of dioecious lineages remains capable of producing a male phenotype in the genetic backgrounds of other lineages within the clade. Nevertheless, the production of a male phenotype in crosses involving pure male plants is consistent with the hypothesis that androdioecy in hexaploid *M. annua* arose as a direct result of hybridisation. In particular, crosses between monoecious tetraploid *M. annua* and male *M. huetii* plants, the putative maternal and paternal progenitors of hexaploid *M. annua* respectively, yielded hybrid progeny with a male phenotype. As the male-determining allele of *M. huetii* appears to be functional in a tetraploid *M. annua* background, it suggests that genes conferring maleness in androdioecious *M. annua* could have been inherited from *M. huetii* directly. The identification of homologous sex-determining genes in *M. huetii* and hexaploid *M. annua* males (see Chapter 3) would be extremely valuable for corroborating this theory, as it remains possible that male-determining genes in hexaploid *M. annua* were derived from tetraploid *M. annua* (potentially via diploid dioecious *M. annua*), or even evolved *de novo*. The *de novo* evolution of a male-determining gene appears unlikely given that hexaploid *M. annua* males were capable of siring male hybrids in crosses with diploid *M. annua*, indicating a conserved basis for sex determination, but it cannot be ruled out; a new locus may have arisen in hexaploid males, for example, which switches on existing (but unused in tetraploid *M. annua*) developmental pathways for the production of males, perhaps by altering hormone levels (see above).

Androdioecy is very rare amongst plants, but in the few species in which it has been studied evidence indicates it has often evolved via dioecy, with selection on females to produce some pollen (Pannell, 2001) e.g. in *Datisca glomerata* (Wolf *et al.*, 2001), *Spinifex littoreus* (Connor, 1996), *Schizopepon bryoniaefolius* (Akimoto *et al.*, 1999) and *Castilla elastica* (Sakai, 2001). The absence of dioecy amongst all other *M. annua* polyploids, including tetraploid *M. annua*, the putative maternal progenitor of the hexaploid lineage, would suggest this is not the case in *M. annua*, however. Instead, the identification of male-determining allele in *M. huetii* capable of producing a male phenotype in lineages across the clade provides strong support for the conjecture that androdioecy in hexaploid *M. annua* arose as a result of hybridisation between monoecious tetraploid *M. annua* and male *M. huetii*. The identification of homologous sex-determining genes in *M. huetii* and hexaploid *M. annua* would nonetheless provide further evidence for this theory.

The absence of males amongst neo-hexaploids generated by artificial polyploidisation of female *M. huetii* x monoecious tetraploid *M. annua* hybrids further supports the theory that androdioecy in hexaploid *M. annua* arose as a result of hybridisation between male *M. huetii* and monoecious tetraploid *M. annua*. Unfortunately, no neo-hexaploids of tetraploid *M. annua* x male *M. huetii* hybrids were produced in this study owing to the low hybridisation success (21.7%, most likely caused by the ineffective emasculation of monoecious plants causing pollen contamination) of this cross and subsequent need to employ an alternative, less efficient method of artificial polyploidisation. With all neo-hexaploids thus of maternal *M. huetii* x paternal tetraploid *M. annua* origin, no putative male-

determining alleles were present in the hybrids used for polyploidisation and the lack of males amongst neo-hexaploids suggests such an allele is necessary for the production of males; males appear unable to segregate simply through hybridisation and polyploidisation of monoecious- and female-determining alleles. Of course, only a very small sample of neo-hexaploids has been observed in this investigation, and the study of a greater number of neo-hexaploids, including those generated from hybrids with a *M. huetii* father, would be extremely valuable for investigating the origin of androdioecy in hexaploid *M. annua*.

Furthermore, it would also be of value to consider the alternative pathways to polyploidy by which the hexaploid *M. annua* lineage was formed. Assuming *M. huetii* and tetraploid *M. annua* were the progenitors of this allopolyploid, hexaploid *M. annua* is most likely to have arisen by either the somatic doubling of a triploid hybrid of the two progenitors, or by the fusion of unreduced (2*n*) gametes from each of the progenitors (Ramsey & Schemske, 1998). In the neo-polyploidisations attempted in this study, I have recreated only the first of these scenarios, but it would also be worthwhile to examine the consequences for sex determination and sex expression of polyploid formation via the fusion of unreduced gametes. For example, polyploidisation is known to influence gene expression levels (Adams & Wendel, 2005), and different pathways to polyploidy may have diverse implications for gene and, consequently, sex expression. The effects of polyploidisation on gene expression is the subject of much research in *Brassica* (Gaeta *et al.*, 2007; 2009), *Senecio* (Hegarty *et al.*, 2005; 2006; 2011) and *Arabidopsis* (Madlung *et al.*, 2005; Wang *et al.*, 2006), yet the effects of different pathways to polyploidy on gene expression remain unconsidered. Ultimately, however, the genetic contribution of

each of the putative progenitors to hexaploid *M. annua* is the same via either pathway, and hence effects on sex expression may be mitigated thus.

5.4.4 The evolution of monoecy in tetraploid *M. annua*

With regard to the evolution of monoecy in tetraploid *M. annua*, all neo-tetraploids produced by artificial polyploidisation of diploid *M. annua*, in addition to all F_1 individuals from crosses amongst neo-tetraploids, remained unisexual. This suggests that genome duplication alone is unable to account for the development of a monoecious phenotype in polyploid *M. annua*. These results are consistent with the model for sex determination in the annual mercuries proposed above, which suggests that alleles promoting axillary male flower production are necessary for the production of a monoecious phenotype, but that these alleles are absent in the genotypes of diploid dioecious *M. annua*. This in turn would suggest that selection for functional hermaphroditism may have been involved in the evolution of monoecy in tetraploid *M. annua*, perhaps to enable neo-polyploids (which likely arose initially at exceptionally low density) to self-fertilise and avoid the fitness costs associated with back-crossing with diploid progenitors and the process of minority cytotype exclusion (Levin, 1975; Rodriguez, 1996; Rausch & Morgan, 2005). Alternatively, if polyploidisation caused a significant decrease in the fitness costs associated with inbreeding, below the threshold at which separate sexes becomes an advantageous trait, functional hermaphroditism, rather than unisexuality, may have become the superior mating strategy in the lineage (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987). Following the alleviation of inbreeding depression, functional hermaphroditism may have been selected in neo-tetraploid *M. annua* to enhance colonisation ability by providing reproductive assurance when colonising new areas

of marginal habitat, thus enabling neo-tetraploid *M. annua* to spread into habitat unoccupied by its dioecious progenitor (Brochmann *et al*., 2004; Pannell *et al*., 2004). Of course, species found in newly colonised habitats may be functionally hermaphroditic not because they have been selected over unisexual species for this particular trait, but simply because they are more likely to be able to establish from a small founder population (and hence more likely to colonise new habitats). Functional hermaphroditism may thus not have evolved in tetraploid *M. annua* as a result of selection for enhanced colonisation ability but for another reason, yet an ability to self-fertilise may also facilitate colonisation in this ruderal species.

If selection for functional hermaphroditism were involved in the evolution of monoecy in tetraploid *M. annua*, genes promoting axillary male flower development (as outlined in the model of sex determination presented above) would have been favoured and spread through the neo-tetraploid population. Although male- and female-sterility genes would have been inherited from diploid *M. annua* at loci homologous to those which determined sex in their progenitor, I can think of no *a priori* reason why genes controlling staminate flower production would necessarily develop at the same loci. These loci would, obviously, have been predisposed to regulating sexual function and thus alleles for the production of a monoecious phenotype could have arisen there (which would make the genetic model proposed above a single-locus, tri-allelic system, with monoecious- and female-determining alleles displaying co-dominance). However, given that hermaphroditic sexual systems are not under the same selective pressures as dioecious systems for genes regulating sexual function to be genetically linked, a mutation that promoted axillary male and female flower development could have arisen at an alternative genetic

locus and spread to fixation through the neo-tetraploid *M. annua* population. Again, the identification of homologous sex-determining genes amongst annual mercuries would be extremely valuable for investigating this question.

The evolution of functional hermaphroditism from separate sexes with polyploidisation is relatively rare amongst plant species, although it has been reported in *Empetrum* (Richards, 1997) and *Isotoma* (McComb, 1968). In these systems, however, shifts in mating system have been speculated to have been caused by disruption of sex determination following polyploidisation, rather than selection for cosexuality. Furthermore, more common amongst plant species is the reverse association; polyploidisation leading to the evolution of dioecy from hermaphroditism in diploid progenitors. This scenario has been reported in at least 12 different genera (Miller & Venable, 2000) and has been attributed to a breakdown in self-incompatibility systems following polyploidisation, with subsequent selection for separate sexes to avoid the costs of inbreeding (Baker, 1984; Miller & Venable, 2000; Charlesworth, 2001). In *Bryonia*, however, a genus comprising monoecious and dioecious polyploids, there is no correlation between sexual system and ploidy level (Volz & Renner, 2008).

Despite the results obtained in this study, it remains possible that genome duplication was directly responsible for the evolution of monoecy in tetraploid *M. annua* by disruption of the species' sex-determining mechanism. For example, changes in allele dosage or expression levels following polyploidisation may have caused such a disruption. In particular, genes regulating hormone production may have precipitated a shift in sex expression; it is well established that phytohormones

play a key role in sex determination in *M. annua* (Louis, 1989; Durand & Durand, 1991) and thus changes in hormone levels could have culminated in the production of a monoecious phenotype. Indeed, Durand (1963) observed monoecious individuals with phenotypes similar to those of wild tetraploids in the progeny of artificial *M. annua* autotetraploids. Artificial polyploids of dioecious *Silene* and *Rumex* species have likewise been reported to produce hermaphroditic progeny (Westergaard, 1958). In this study, the low efficiency of the colchicine-induced polyploidisation method, coupled with the low fertility of the few neo-polyploids obtained, may have meant that insufficient neo-tetraploids were produced for a monoecious phenotype to have been observed; Durand (1963) had greater polyploidisation success, enabling the gender of more individuals to be scored. Further study of a greater number of neo-polyploids would thus be worthwhile to fully address whether monoecy in tetraploid *M. annua* might have arisen as a direct consequence of genome duplication, or as a result of selection for functional hermaphroditism.

5.4.5 The effects of hybridisation on fertility and morphology

Hybrid fertility, both pollen viability and the proportion of female flowers setting seed, was significantly lower than that of individuals from parent populations. This result is not surprising; viable gamete production is typically inhibited in hybrids as differences in chromosome number and structure prevent pairing and segregation during meiosis (Sybenga, 1975), whilst hybrid sterility has been implicated in the movement of the diploid-hexaploid *M. annua* contact zones in Spain, (Buggs & Pannell, 2006). Interestingly, female fertility of *M. huetii* and diploid *M. annua* hybrids was elevated relative to that of other hybrids (all of which were inter-ploidy

hybrids), perhaps due to the similar genome size and architecture of these sister species which permitted a degree of meiosis to occur. There was no significant difference in the pollen viability of any of the hybrids, however, suggesting differences in the genome size of progenitors does not influence male fertility. The fertility of neo-hexaploids produced by in vivo polyploidisation of female *M. huetii* x monoecious tetraploid *M. annua* triploid hybrids is also noteworthy; triploid hybrids had exceptionally low female fertility, but in neo-polyploids fertility was apparently restored as over 100 viable seeds were collected per plant, demonstration of the escape genome duplication can provide from the failure of meiosis.

Finally, hybrid morphology was highly similar to that of individuals from parent lineages. Discriminant function analyses were frequently unable to classify hybrids correctly, with polyploid lineages and hybrids most difficult to distinguish between. With morphological analyses of individuals from parent populations struggling to distinguish reliably between tetraploid and hexaploid *M. annua* plants, it is unsurprising that hybrids were similarly difficult to classify. *M. huetii* was clearly distinguishable by its smaller size, leaves and narrower stems, whilst hybrids of *M. huetii* and diploid *M. annua* were likewise discernible by their intermediate morphology relative to their progenitors. Interestingly, polyploid hybrids were only ever misclassified as tetraploid or hexaploid *M. annua*, and never as diploid *M. annua* or *M. huetii*, the greater morphological similarity between polyploid hybrids and polyploid progenitors perhaps attributable to increased relative gene contribution from these parents. In general, however, the morphological similarity of hybrids and parent lineages is expected given the similar morphology of the various lineages of annual mercuries. In only one case were hybrids found to display more extreme

morphological traits than either parent; hybrids of diploid *M. annua* and tetraploid *M. annua* had greater vegetative mass and possessed larger leaves relative to their progenitors. This is somewhat surprising given the relatedness of diploid and tetraploid *M. annua*, and that all other hybrids displayed morphology intermediate to that of their progenitors. The more extreme morphology occurred presumably as a result of interactions between the genomes of the parent lineages, with differences in allele dosage and gene expression patterns in hybrids potentially giving rise to more extreme phenotypes than either parent. Hybrids of *Helianthus* species have been reported to possess phenotypes more extreme than those of parental lineages, which evidence suggests was an important source of novel variation in adapting to new niches (Lexer *et al.*, 2003; Rieseberg *et al.*, 2003b; 2007). In *M. annua*, differences have been observed in the drought response of diploid and hexaploid *M. annua* in terms of their stomatal characteristics, photosynthesis and carbon isotope discrimination (Buggs, 2004). It is possible that such variation may have been precipitated by hybridisation, with the coming together of divergent tetraploid *M. annua* and *M. huetii* genomes in allohexaploids causing variation in traits which enabled them to colonise the more water-limited Iberian peninsula. Tetraploid *M. annua* (restricted to southern Morocco) also occupies more water-limited environments relative to its diploid progenitor, with it possible that polyploidisation likewise enabled the colonisation of such environments (although its preponderance in more water-limited environments relative to diploid *M. annua* has also been attributed to its monoecious sexual system and ability to self-fertilise; see Pannell, 1997b). These ideas remain speculative, although as well as being a useful model system in the study of sex determination, *M. annua* may also have the potential for

use in studies of the effects of polyploidisation on gene expression and local adaptation.

5.4.6 Conclusions and future work

The evidence presented in this chapter indicates a conserved mechanism of single-locus, active-Y genetic sex determination across the annual mercury clade. The inheritance of a single male-determining allele gives rise to male progeny in all lineages of annual mercuries, regardless of the allele's origin. Female or hermaphroditic hybrid progeny may also be produced in crosses between annual mercury lineages, depending on the inheritance of alleles controlling axillary male flower development. Based on estimates of sex allocation in hermaphroditic hybrids, monoecious-determining alleles show partial, though not full, dominance over female-determining alleles. The presence of male-determining gene(s) functional in lineages across the annual mercury clade is also consistent with the hypothesis that androdioecy in hexaploid *M. annua* arose as a direct result of hybridisation between monoecious tetraploid *M. annua* and male *M. huetii*.

The identification of a conserved mechanism of sex determination across the annual mercury clade is an exciting result. Genes regulating male and female sexual expression remain functionally active in the genetic backgrounds of other lineages within the clade, in spite of differences in sexual system. The identification by molecular methods of homologous genes or gene transcripts for sex determination, or homologous sex-linked markers, amongst annual mercury species would provide further information on the extent of the conservation of sex determination and may shed light on the sexual-system transitions which have occurred across the clade, the

specific role hybridisation and polyploidisation have played in those transitions and the implications of said transitions for sex-determining mechanisms. Such an investigation was attempted in Chapter 3 of this thesis, in which the male-linked DNA marker *OPB01-1562* from diploid *M. annua* was found not to be conserved across the annual mercury clade. Nonetheless, the results presented in this chapter suggest the male-determining allele of diploid *M. annua* is functional in other lineages of the clade and that such homologies in sex-determining genes may exist. It is therefore, I believe, an avenue of study worthy of future pursuit in order to provide conclusive evidence of a conserved system of sex determination amongst the annual mercuries.

The incorporation of *M. canariensis* in hybridisations would also be of interest with regard to studying the conservation of sex-determining mechanisms across the annual mercury clade. *M. canariensis* is unique amongst the annual mercuries in that it is a dioecious polyploid and, given that it has an unknown species as its paternal progenitor, it would be interesting to examine whether male-determining alleles from other lineages remain functional in *M. canariensis*, as well as whether the sex-determining mechanism of *M. canariensis* is functional in other annual mercuries, especially given the potential introgression of male-determining genes from outside the annual mercury clade. Study and comparison of the sex-determining mechanisms of other *Mercurialis* species from outside the annual clade would be similarly interesting for assessing the conservation of sex determination across the genus as a whole, especially given that dioecy is potentially ancestral in the genus (Durand & Durand, 1992; Krähenbühl *et al.*, 2002; Obbard *et al.*, 2006b). For example, *M. perennis* is dioecious and often polyploid, whilst *M. leiocarpa*

polyploids display both monoecy and dioecy (Krähenbühl & Küpfer, 1995), potentially enabling comparisons of the effects of polyploidisation on sexual system and sex determination to be made with *M. annua*.

In addition to further hybridisation analyses, greater study of the immediate effects of polyploidisation on the sex expression and sex determination of annual mercuries would also be worthwhile. In particular, inducing polyploidisation in hybrid progeny from crosses between tetraploid *M. annua* and male *M. huetii* (by employing a more efficient system of hybridisation and polyploidisation) would enable artificial reconstruction of this allopolyploid and examination of whether male neo-hexaploids are produced, further improving our understanding of how the rare mating system androdioecy arose in hexaploid *M. annua*. Furthermore, generating a greater number of neo-tetraploids from diploid *M. annua* seed and observing the segregation of gender through multiple generations of neo-tetraploids to examine whether monoecious phenotypes do appear, perhaps in combination with molecular analyses of previously identified sex-linked genes, would permit a more detailed analysis of whether genome duplication might have precipitated the evolution of monoecy in tetraploid *M. annua* directly.

The specific implications for sex-determining mechanisms of hybridisation and polyploidisation in the annual mercury clade thus remain an interesting puzzle. Molecular analysis of sex-determining genes in lineages across the clade is most likely to provide answers to questions regarding the consequences for sex determination of hybridisation, polyploidisation and sexual-system transitions.

6. ENVIRONMENTAL SEX DETERMINATION IN ANDRODIOECIOUS *MERCURIALIS ANNUA*

6.1 INTRODUCTION

Environmental sex determination (ESD) occurs when the gender of an individual is determined at a point after fertilization according to the level of one or more environmental factors (Bull, 1983). Unlike genetic sex determination (GSD), under ESD an individual's genotype has no influence on its gender (although sexual development is not supposed to occur without the involvement of genes and recent thinking emphasises the fact that both genetic and non-genetic factors are typically involved in sex determination to some extent; Bull, 1983; Uller & Helanterä, 2011). ESD is much less common than GSD across the natural world, but is found in a diverse range of taxa, including nematodes (Petersen, 1972; Blackmore & Charnov, 1989), fish (Baroiller *et al*., 2009) and reptiles (Janzen & Paukstis, 1991). Reptilian groups have been particularly well studied, with temperature-dependent sex determination, when sex is determined by ambient temperatures during incubation, widespread amongst turtles, lizards and crocodilians (Janzen & Paukstis, 1991; Valenzuela & Lance, 2004).

Amongst plants, ESD has been identified in a number of species, including the homosporous ferns *Ceratopteris richardii* and *Woodwardia radicans*, in which gametophytes develop as males only in the presence of antheridiogen, a hormone secreted into the soil by hermaphrodites (and females in the case of *W. radicans*), hence male frequencies are heavily influenced by population structure (Banks *et al*.,

1993; Korpelainen, 1998; Quintanilla *et al*., 2007). Rather than 'full ESD', where an individual's qualitative gender is determined by the environment, the most common form of ESD in plants is sexual lability, or gender plasticity, where sexual expression, and hence quantitative gender, shift over an individual's lifetime according to some environmental factor (Korpelainen, 1998; Delph & Wolf, 2005). For example, in the epiphytic orchid *Catasetum viridiflavum*, gender is determined largely by light intensity, with females predominating in open canopies with high light levels, males prevailing in closed, darker canopies and hermaphrodites occurring as the result of changes in sex expression within years (Zimmerman, 1991). Similarly, a study by Freeman *et al*. (1981) observed an increase in the ratio of female to male flowers between dry and mesic sites amongst individuals of the monoecious species *Juniperus osteosperma*, *Quercus gambelii* and *Sarcobatus vermiculatus*. Water stress has also been implicated in shifts in the ratio of male to female inflorescences in the African oil palm, *Elaeis guineensis* (Adam *et al*., 2011). The conditions under which mechanisms of ESD, including the above, are predicted to evolve can be summarised by the Charnov-Bull model (Charnov & Bull, 1977).

The Charnov-Bull model (Charnov & Bull, 1977) predicts that ESD should be favoured over GSD in a species when three conditions are met: i) an individual's fitness (as a male or female) is strongly influenced by environmental conditions; ii) the environmental conditions influencing fitness are spatially or temporally heterogeneous; and iii) an individual has little control over which environment it will experience. This theory can be applied to sex expression in functional hermaphrodites (such as monoecious plants) as well as to species with separate sexes (Charnov & Bull, 1977). Factors contributing to environmental heterogeneity in

terms of the relative fitness of being male or female may include: local mate competition; resource availability; and sex-specific mortality (Charnov & Bull, 1977). Given the sessile nature of plants, we might expect ESD to be relatively common amongst plant species, perhaps explaining the relatively high number of species which display a degree of sexual lability (Korpelainen, 1998). In hexaploid androdioecious populations of *Mercurialis annua*, sex determination has been reported to possess an environmental component, namely growing density, which influences both the proportion of male individuals in the population and the relative allocation to male and female reproductive function in monoecious hermaphrodites (Pannell, 1997a; 1997b).

Gender in androdioecious *M. annua* has been reported to be controlled by a mixed genetic-environmental system of sex determination (Pannell, 1997a; 1997b). From the results of a breeding experiment, Pannell (1997a) concluded that males are determined by the inheritance of a dominant allele at a single locus. However, in a controlled glasshouse experiment and from field observations, Pannell (1997a; 1997b) observed that the frequency of males was also correlated positively with growing density, indicating a mechanism of density-dependent gender choice. Furthermore, under controlled conditions, the allocation of resources to pollen production relative to seed production (sex allocation) was found to decrease in hermaphroditic (monoecious) individuals with increasing density, i.e. there was a significant shift in sex allocation towards female reproductive function with increasing density (Pannell, 1997b; Dorken & Pannell, 2008). Sex allocation in hexaploid *M. annua* hermaphrodites has also been reported to be more female-biased in nutrient-poor soils and under intra-specific competition (Hesse & Pannell, 2011b).

In identifying an environmental component to sex determination in hexaploid *M. annua*, Pannell's work revealed the existence of 'late cosexes' in the species, hermaphroditic individuals that possess male pedunculate inflorescences in their lower leaf axils (Pannell, 1997a). Pannell interpreted these individuals as having initially developed as males, but switched to become hermaphroditic late in their development. The frequency of late cosexes correlated negatively with density, corresponding to the increase in the frequency of males. As the frequency of 'pure cosexes' (hermaphrodites with axillary male flowers) was independent of density, Pannell concluded that only males were capable of sex change, switching to become hermaphroditic at low densities (Pannell, 1997a). This conclusion was also supported by the work of a breeding experiment, in which late cosexes occurred only in the progeny of hermaphroditic individuals crossed with males and never amongst hermaphrodite-sired progeny (Pannell, 1997a). Sex-specific mortality and differential germination between the sexes were ruled out by Pannell's experimental design (Pannell, 1997a).

Pannell (1997a; 1997c) has suggested that the environmental component to sex determination in hexaploid androdioecious *M. annua* evolved as an adaptive response to selection for reproductive assurance during colonisation. Androdioecy is an extremely rare breeding system which is thought unlikely to be maintained in nature (Lloyd, 1975; Charlesworth & Charlesworth, 1978a; Charlesworth, 1984; Fritsch & Rieseberg, 1992; Pannell, 1997c; Pannell, 2002). In order for males to coexist with functional hermaphrodites, they must be at least twice as fertile as the male reproductive function of the hermaphrodites, a factor which increases with

increasing selfing rates amongst the hermaphrodites (Lloyd, 1975; Charlesworth & Charlesworth, 1978a; Charlesworth, 1984; Pannell, 2002). Furthermore, in a colonising species such as *M. annua*, self-fertile hermaphrodites benefit from the advantage of reproductive assurance over males when founding a new population (Baker, 1955; Jarne & Charlesworth, 1993; Pannell, 1997c). The ability of *M. annua* males to alter their sex expression and function cosexually at low densities will mitigate against selection for reproductive assurance, whilst still permitting males to enjoy high outcrossing rates when functioning as pure males in high density populations (Pannell, 1997a; 1997c). The shift towards increased 'femaleness' in monoecious hermaphrodites at high density may likewise be explained as a response to the local mating environment, or, alternatively, as a response to changes in resource availability as a result of intra-specific competition (Pannell, 1997b; Dorken & Pannell, 2008; Hesse & Pannell, 2011b).

The work of Pannell thus strongly indicates there to be an environmental component to sex determination in hexaploid androdioecious *M. annua*. However, further studies (Pannell, 1997b; Sánchez-Vilas & Pannell, 2012; Russell & Pannell, unpublished data) have failed to replicate these findings and have not detected any change in the frequency of males with density. Moreover, it is unclear how the mixed genetic-environmental system of sex determination proposed by Pannell may be corroborated with the results presented in the previous chapters of this thesis, which indicate that gender is determined purely genetically by a single-locus mechanism, with male heterogamety, across the annual mercury clade (see Chapters 2 & 5). It is therefore important to examine further the findings of Pannell in order to establish whether there is indeed an environmental component to sex

determination in this lineage and, if present, how the potentially ubiquitous single-locus genetic mechanism of sex determination in the annual mercury clade has been modified to enable individuals to respond to growing density and whether other annual mercury lineages also possess such a capacity for density-dependent gender choice.

One explanation to account for the findings of Pannell is that there exists geographic variation in the capacity for density-dependent gender choice in the lineage. Recent studies which have failed to detect changes in the frequency of males with density have used populations from across the range of androdioecious *M. annua* (Sánchez-Vilas & Pannell, 2012; Russell & Pannell, unpublished data), but have not used the populations in and around Seville, southern Spain, used by Pannell (1997a; 1997b) and which are now, unfortunately, locally extinct. Given the evolutionary history of hexaploid *M. annua*, which is believed to have undergone postglacial range expansion from a north African or southern Iberian refugium and exhibits a latitudinal gradient in genetic diversity (Obbard *et al.*, 2006c), it is possible that geographic or population-level variation exists in the species' sex-determining mechanism. I here thus examine populations from across the geographic range of hexaploid androdioecious *M. annua* to test for variation in the response of individuals to density in terms of their sex determination and sex allocation.

In this chapter, I test for the presence of an environmental component to sex determination in hexaploid androdioecious *M. annua* in two experiments. First, I examine whether male frequency, the frequency of hermaphrodites bearing male pedunculate inflorescences, or sex allocation are influenced by growing density in

three populations of hexaploid *M. annua* sampled from across the geographic range of the species. Second, I focus my study upon the presence of ESD in three Portuguese populations of hexaploid *M. annua* which, from the results of the first experiment, appear to display a degree of density-dependent gender choice. I likewise examine whether male frequency, pedunculate hermaphrodite frequency, or sex allocation are influenced by growing density in individuals of these populations.

6.2 MATERIALS AND METHODS

6.2.1 Experiment 1: testing the effects of growing density on the sex ratios and sex allocation of three geographically distant populations

The population of hexaploid androdioecious *M. annua* studied by Pannell (1997a) has become locally extinct and was thus unable to be further examined in this study. Instead, seeds for this experiment were collected from three wild populations sampled from across the lineage's geographic range, specifically: Ksar el-Kebir (Morocco); Trebujena (Spain); and Monchique (Portugal; see Appendix 9.1 for full details of population locations). Seeds from each population were collected from 20-30 large hermaphroditic plants and mixed thoroughly to create bulk seed stocks. In late summer 2010, seeds from each population were sown in soil-based compost in separate 8 cm diameter pots. For each population, 12 seeds were sown into each of 225 pots (giving 675 pots sown in total). Immediately following germination, when seedlings were still at the cotyledon stage, the number of plants in each pot was thinned to achieve four density levels. For each population, densities were manipulated as follows: 120 pots with one plant; 60 pots with two plants; 30 pots with four plants; and 15 pots with eight plants. The same number of plants was thus grown at each density level for each population to enable sex ratios to be estimated with equal precision across all population x density treatment combinations, with analyses accounting for the nesting of plants within pots (see below). The density levels created in this experiment were selected as being representative of natural variation in the population density of androdioecious *M. annua*; densities of wild populations have been observed to range from just a single plant per population to > 1,300 plants per m^2 (Pannell, 1995; 1997b), and thus densities of a single plant per 8 cm diameter pot up to eight plants per pot (which corresponds to approximately

1,600 plants per m^2) were deemed to correspond to natural variation in density. The 675 pots were divided into three blocks, with an equal number of pots of each population x density treatment combination assigned to each of the blocks. Each block was located on a separate bench within the same glasshouse and pots were arranged randomly within blocks. Designated densities were maintained over the course of the experiment by the removal of any late emergents.

All plants were watered and maintained under standard glasshouse conditions for a period of 12 weeks, following which all individuals were harvested and scored for sex as either a male, 'axillary hermaphrodite' or 'pedunculate hermaphrodite'; in this study, the term 'pedunculate hermaphrodite' was used to classify all hermaphroditic individuals which possessed male flowers borne on axillary peduncles anywhere on the plant, whilst all hermaphrodites bearing male and female flowers solely in leaf axils were referred to as 'axillary hermaphrodites' (Figure 6.1). This terminology was adopted in preference to Pannell's 'late cosex' classification (which refers specifically to individuals with "male pedunculate inflorescences in their lower leaf axils"; Pannell, 1997a) since, in this study, many hermaphrodites were observed to possess male flowers on peduncles, but these peduncles varied widely in length, frequency and location on the plant. Alternative terminology was therefore used to simplify classification and better encompass the diversity observed in hermaphrodite floral morphology.

Reproductive effort and sex allocation were also estimated in nine males and nine axillary hermaphrodites selected at random from each population x density treatment combination (three of each sex sampled randomly per block). Total above-ground

dry vegetative biomass and the dried biomass of male and female flowers were separately measured for each of these individuals and estimates of reproductive effort and sex allocation calculated as described in section 5.2.2 of this thesis.

6.2.2 Experiment 2: testing the effects of growing density on the sex ratios and sex allocation of three Portuguese populations

Following analysis of the effects of density on the sex ratios of three geographically distant populations in Experiment 1, the sex ratios of the population from Monchique (Portugal) were found to display significant variation with density, unlike the populations sampled from Morocco and Spain. A second experiment was therefore undertaken focusing upon the effects of density on sex ratios and sex allocation in three Portuguese populations of hexaploid androdioecious *M. annua*. Seeds for this experiment were bulk collected (using an identical procedure to that described in Experiment 1) from three populations in southern Portugal, located at Lagos, Portelas and Monchique (N.B. the population sampled from Monchique is a different population from that used in Experiment 1; Appendix 9.1). In summer 2011, seeds from each population were sown following a similar experimental design to Experiment 1, but with several modifications. For each population, 20 seeds were initially sown into each of 248 pots (giving 744 pots sown in total). Following germination, plants were thinned to create five density levels as follows: 128 pots with one plant; 64 pots with two plants; 32 pots with four plants; 16 pots with eight plants; and 8 pots with sixteen plants. An additional density level of sixteen plants per pot was included in this experiment in order to assess whether the variation in sex ratios with density detected in Experiment 1 was maintained at exceptionally high density levels. The 744 pots were divided into four blocks, again

with an equal number of pots of each population x density treatment combination assigned to each block, and pots were randomised within blocks.

All plants were watered and maintained for a period of nine weeks, at which point all individuals were harvested and scored for sex as either a male, axillary hermaphrodite or pedunculate hermaphrodite. Reproductive effort and sex allocation were also estimated, as described for Experiment 1, in eight to ten males and eight to ten hermaphrodites selected randomly from each population x density treatment combination. The hermaphrodites sampled consisted of a random selection of axillary and pedunculate individuals, with the presence or absence of peduncles recorded for each.

6.2.3 Data analysis

Generalized linear mixed effects models (GLMMs) were used to analyse whether the proportion of males, axillary hermaphrodites and pedunculate hermaphrodites were influenced by growing density and whether there were differences in the effect of growing density between populations (i.e. whether there was a population x density interaction) in both experiments. The proportion of each sexual phenotype per pot (e.g. number of males/total number of individuals) was used as the response variable, with population fitted as a fixed factor, density fitted as a covariate and block fitted as a random factor, specifying a binomial error structure (full model: PROPORTION MALES = BLOCK + POPULATION | DENSITY).

Linear mixed effects models (LMEs) were used to analyse the effects of density on reproductive effort and sex allocation and whether there were differences in the

effect of density between populations (i.e. whether there was a population x density interaction) in Experiment 1. As measures of reproductive effort and sex allocation were highly bimodal in distribution (due to two classes of sex being sampled), the dataset was split into two according to sex and LMEs used to test for the effects of density and population on these measures separately in males and hermaphrodites. In each case, one of total reproductive effort (TRE), male reproductive effort (MRE), female reproductive effort (FRE) or sex allocation was set as the response variable, with population fitted as a fixed factor, density fitted as a covariate and block fitted as a random factor (full model: TRE = BLOCK + POPULATION | DENSITY). Similar LMEs were also used to analyse the effects of density and population on reproductive effort and sex allocation in Experiment 2. Furthermore, LMEs were also used to analyse differences in the reproductive effort and sex allocation of pedunculate versus axillary hermaphrodites, and whether there were differences in the effects of density on each (i.e. whether there was a peduncle x density interaction) in this experiment. In such analyses, one of TRE, MRE, FRE or sex allocation was again set as the response variable, with population and peduncle presence fitted as fixed factors, density fitted as a covariate and block fitted as a random factor (full model: TRE = BLOCK + POPULATION | DENSITY + PEDUNCLE | DENSITY). For all LMEs, response variables were square-root or $\log_{10}$ transformed where necessary in order to satisfy the assumptions of normality and homogeneity of variance. All analyses were conducted in *R*, version 2.8.0 (http://www.r-project.org).

Figure 6.1 Photographs of apices of hexaploid *M. annua* individuals of the different sexual phenotypes scored in this study: a. male; b. axillary hermaphrodite; and c. pedunculate hermaphrodite. 2d. shows a peduncle from a pedunculate hermaphrodite. Note the difference in the peduncles of males and pedunculate hermaphrodites; peduncles in males are significantly longer and bear several clusters of male flowers compared to those of pedunculate hermaphrodites.

6.3 RESULTS

6.3.1 Sex ratio variation in Experiment 1

The proportion of males did not differ significantly with growing density in any of the three populations tested in Experiment 1 ($\chi^2_1 = 0.84$, $p = 0.360$; Table 6.1; Table 6.2; Figure 6.2). In contrast, the proportions of axillary and pedunculate hermaphrodites were both significantly influenced by density. In the case of axillary hermaphrodites, there was a significant population x density interaction, with the proportion of axillary hermaphrodites positively correlated with density in the Portuguese population, but unaffected by density in either the Moroccan or Spanish populations ($\chi^2_2 = 10.5$, $p = 0.005$). Variation in the proportion of pedunculate hermaphrodites with density was unable to be tested in the Moroccan and Spanish populations as an exceptionally low number of pedunculate hermaphrodites were produced in these populations (≤ 3 individuals per density level). The proportion of pedunculate hermaphrodites was negatively correlated with density in the Portuguese population, however, ($\chi^2_1 = 8.99$, $p = 0.003$; Figure 6.2), where the frequency of this sexual phenotype was much greater.

6.3.2 Sex ratio variation in Experiment 2

The proportion of males was not significantly influenced by growing density in any of the three populations tested in Experiment 2 ($\chi^2_1 = 0.25$, $p = 0.615$; Table 6.3; Table 6.4; Figure 6.3). The proportions of axillary and pedunculate hermaphrodites scored did, however, differ significantly with density. The proportion of axillary hermaphrodites was found to increase with density ($\chi^2_1 = 62.4$, $p < 0.001$), whilst, conversely, the proportion of pedunculate hermaphrodites decreased with density ($\chi^2_1 = 102.7$, $p < 0.001$). This trend was detected in all three populations studied in

this experiment, with all population x density interactions non-significant (Table 6.4).

6.3.3 Reproductive effort and sex allocation in Experiment 1

Total reproductive effort was negatively correlated with density in males from the Moroccan and Spanish populations, but was uninfluenced by density in males from the Portuguese population ($F_{2, 100} = 3.63$, $p = 0.030$; Table 6.5). Conversely, hermaphrodites from the Portuguese population increased reproductive effort with increasing density, whilst Moroccan hermaphrodites allocated less to reproduction and in Spanish hermaphrodites total reproductive effort was unaffected by density, although this effect was only marginally significant ($F_{2, 100} = 2.85$, $p = 0.062$). In all three populations, however, male reproductive effort in hermaphrodites was strongly negatively correlated with density ($F_{1, 102} = 40.3$, $p < 0.001$). The effects of density on female reproductive effort were more variable between populations: FRE increased with density in the Portuguese population and (to a lesser extent) in the Spanish population, but was uninfluenced by density in the Moroccan population ($F_{2, 100} = 3.36$, $p = 0.039$). Sex allocation in hermaphrodites therefore became quantitatively more female-biased with increasing density in all three populations ($F_{1, 102} = 36.2$, $p < 0.001$).

6.3.4 Reproductive effort and sex allocation in Experiment 2

In Experiment 2, total reproductive effort was unaffected by density in males ($F_{1, 132} = 1.79$, $p = 0.184$; Table 6.6) but was positively correlated with density in hermaphrodites ($F_{1, 128} = 9.48$, $p = 0.003$) in all three populations. Male reproductive effort in hermaphrodites decreased with density in populations from

Portelas and Lagos, but was uninfluenced by density in the population sampled from Monchique ($F_{2, 126} = 4.84$, $p = 0.009$). Female reproductive effort, however, was strongly positively correlated with density in hermaphrodites from all populations ($F_{1, 128} = 19.5$, $p < 0.001$), leading sex allocation to become quantitatively more female-biased with increasing density in all three populations ($F_{1, 128} = 18.6$, $p < 0.001$; Figure 6.4), although this effect was weaker in the Monchique population, with the interaction term marginally significant ($F_{2, 126} = 3.13$, $p = 0.047$).

Focusing on the effect the presence of peduncles had on reproductive effort and sex allocation in hermaphrodites, all peduncle x density interactions were non-significant (Table 6.6), indicating that there were no differences in the effects of density on axillary and pedunculate hermaphrodites. Pedunculate hermaphrodites did, however, allocate significantly fewer resources to reproduction compared to axillary hermaphrodites ($F_{1, 128} = 8.23$, $p = 0.005$). Furthermore, male reproductive effort was marginally significantly different between the two, with pollen production greater in pedunculate hermaphrodites ($F_{1, 126} = 3.32$, $p = 0.071$), whereas female reproductive effort was significantly greater in axillary hermaphrodites ($F_{1, 128} = 13.1$, $p < 0.001$). Pedunculate hermaphrodites subsequently had a quantitatively more male-biased sex allocation compared to axillary hermaphrodites ($F_{1, 128} = 8.20$, $p = 0.005$).

Table 6.1 The total number of males, axillary hermaphrodites and pedunculate hermaphrodites scored for each population x density treatment combination in Experiment 1. The total number of plants harvested for each treatment combination varies due to the omission, or reallocation to the appropriate density class, of pots in which the observed density differed from that intended. See Appendix 9.1 for full details of the populations from which seeds were sampled for this experiment.

Population	Density	Males	Axillary Hermaphrodites	Pedunculate Hermaphrodites	Total Plants
Morocco, MOR1	1	32	88	1	121
Morocco, MOR1	2	52	63	3	118
Morocco, MOR1	4	45	72	3	120
Morocco, MOR1	8	52	66	2	120
Portugal, 1525a	1	38	63	19	120
Portugal, 1525a	2	32	72	16	120
Portugal, 1525a	4	40	69	11	120
Portugal, 1525a	8	30	84	6	120
Spain, 1053a	1	23	95	3	121
Spain, 1053a	2	23	93	2	118
Spain, 1053a	4	19	98	3	120
Spain, 1053a	8	26	92	2	120

Table 6.2 Results of the generalized linear mixed effects models (GLMMs) analysing the effects of density and population on the proportion of males, axillary hermaphrodites and pedunculate hermaphrodites produced in Experiment 1. Variation in the proportion of pedunculate hermaphrodites with density was unable to be tested in the Moroccan and Spanish populations as an exceptionally low number of pedunculate hermaphrodites were produced in these populations; the results presented here from analyses of the effects of density on the proportion of pedunculate hermaphrodites are hence from the Portuguese population only. Values significant at $p < 0.05$ are shown in bold.

		Proportion of males		**Proportion of axillary hermaphrodites**		**Proportion of pedunculate hermaphrodites**	
	d.f.	χ^2	***p***	χ^2	***p***	χ^2	***p***
Population	2	42.3	**< 0.001**	52.7	**< 0.001**	NA	NA
Density	1	0.84	0.360	0.06	0.804	8.99	**0.003**
Population x Density	2	3.60	0.166	10.5	**0.005**	NA	NA

Table 6.3 The total number of males, axillary hermaphrodites and pedunculate hermaphrodites scored for each population x density treatment combination in Experiment 2. The total number of plants harvested for each treatment combination varies due to the omission, or reallocation to the appropriate density class, of pots in which the observed density differed from that intended. See Appendix 9.1 for full details of the populations from which seeds were sampled for this experiment.

Population	Density	Males	Axillary Hermaphrodites	Pedunculate Hermaphrodites	Total Plants
Lagos, 1521a	1	34	36	56	126
Lagos, 1521a	2	52	48	30	130
Lagos, 1521a	4	51	48	29	128
Lagos, 1521a	8	44	58	18	120
Lagos, 1521a	16	43	71	14	128
Monchique, 1522a	1	22	31	72	125
Monchique, 1522a	2	21	51	60	132
Monchique, 1522a	4	31	51	42	124
Monchique, 1522a	8	28	59	33	120
Monchique, 1522a	16	30	75	23	128
Portelas, 1520a	1	19	76	33	128
Portelas, 1520a	2	26	78	22	126
Portelas, 1520a	4	29	82	17	128
Portelas, 1520a	8	17	88	7	112
Portelas, 1520a	16	22	99	7	128

Table 6.4 Results of the generalized linear mixed effects models (GLMMs) analysing the effects of density and population on the proportion of males, axillary hermaphrodites and pedunculate hermaphrodites produced in Experiment 2. Values significant at $p < 0.05$ are shown in bold.

		Proportion of males		**Proportion of axillary hermaphrodites**		**Proportion of pedunculate hermaphrodites**	
	d.f.	χ^2	***p***	χ^2	***p***	χ^2	***p***
Population	2	56.1	**< 0.001**	120.1	**< 0.001**	94.3	**< 0.001**
Density	1	0.25	0.615	62.4	**< 0.001**	102.7	**< 0.001**
Population x Density	2	1.70	0.427	0.45	0.800	0.32	0.853

Table 6.5 Results of the linear mixed effects models (LMEs) analysing the effects of density and population on reproductive effort and sex allocation in males and axillary hermaphrodites harvested in Experiment 1. TRE = total reproductive effort; MRE = male reproductive effort; FRE = female reproductive effort. Error *d.f.* = 100 for full models and adjusted accordingly for main effects where interaction is non-significant. Values significant at $p < 0.05$ are shown in bold.

		TRE (males)		**TRE (hermaphrodites)**		**MRE (hermaphrodites)**		**FRE (hermaphrodites)**		**Sex allocation (hermaphrodites)**	
	d.f.	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***
Population	2	8.89	**< 0.001**	5.55	**0.005**	18.3	**< 0.001**	5.40	**0.006**	14.9	**< 0.001**
Density	1	18.1	**< 0.001**	0.01	0.941	40.3	**< 0.001**	4.21	**0.043**	36.2	**< 0.001**
Population x Density	2	3.63	**0.030**	2.85	0.062	2.69	0.073	3.36	**0.039**	1.82	0.167

Table 6.6 Results of the linear mixed effects models (LMEs) analysing the effects of density, population and the presence of peduncles on reproductive effort and sex allocation in males and hermaphrodites harvested in Experiment 2. TRE = total reproductive effort; MRE = male reproductive effort; FRE = female reproductive effort. Error *d.f.* = 130 for full model analysing males and = 125 for full model analysing hermaphrodites and adjusted accordingly for main effects where interaction(s) are non-significant. Values significant at $p < 0.05$ are shown in bold.

		TRE (males)		**TRE (hermaphrodites)**		**MRE (hermaphrodites)**		**FRE (hermaphrodites)**		**Sex allocation (hermaphrodites)**	
	d.f.	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***
Population	2	2.20	0.115	10.4	**< 0.001**	1.27	0.284	4.61	**0.012**	1.77	0.175
Density	1	1.79	0.184	9.48	**0.003**	8.22	**0.005**	19.5	**< 0.001**	18.6	**< 0.001**
Peduncle Presence	1	NA	NA	8.23	**0.005**	3.32	0.071	13.1	**< 0.001**	8.20	**0.005**
Population x Density	2	0.26	0.772	1.49	0.230	4.84	**0.009**	0.63	0.537	3.13	**0.047**
Peduncle x Density	1	NA	NA	0.19	0.661	0.41	0.524	1.02	0.314	0.91	0.342

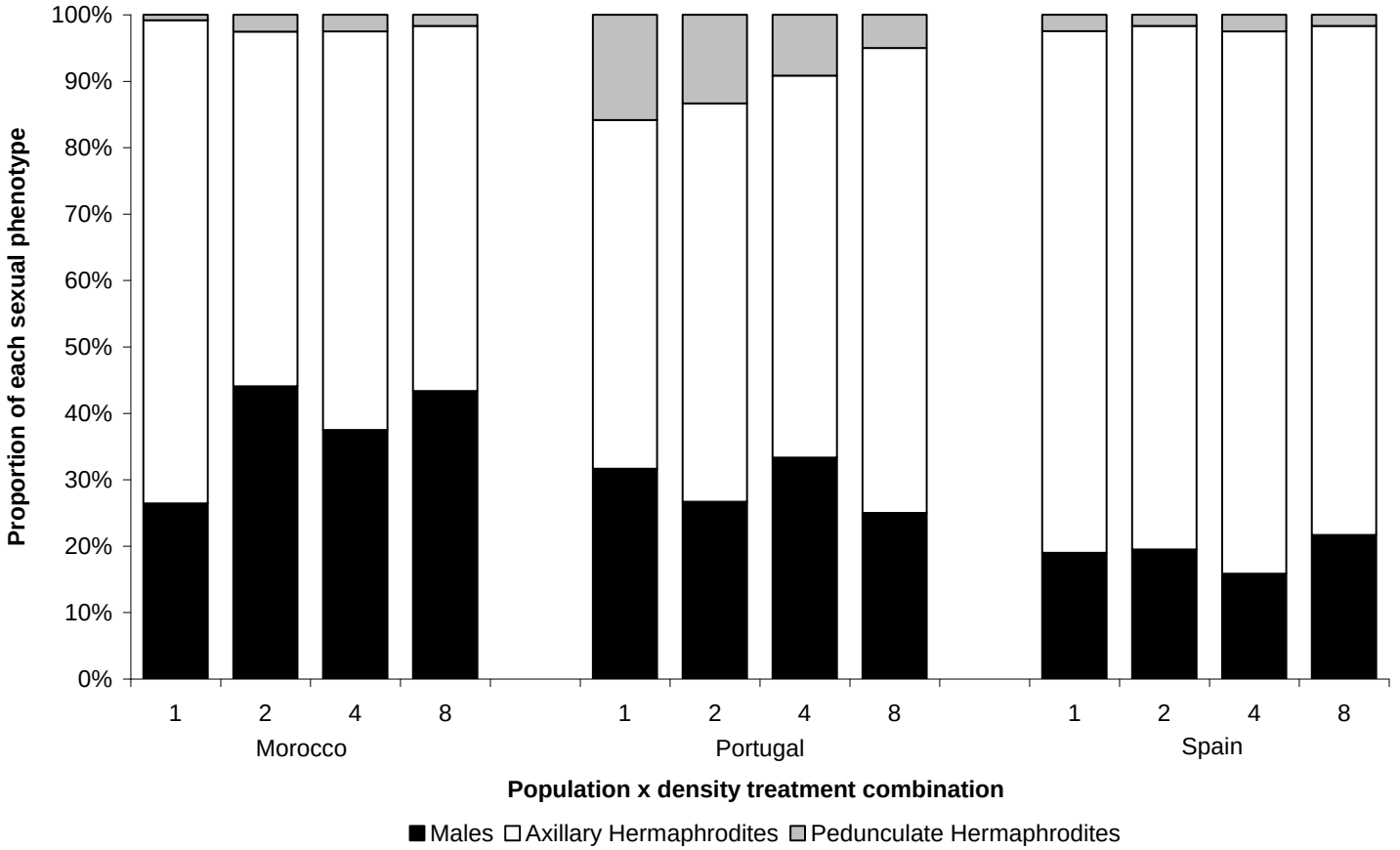


Figure 6.2 The proportion of males, axillary hermaphrodites and pedunculate hermaphrodites scored for each population x density treatment combination in Experiment 1.

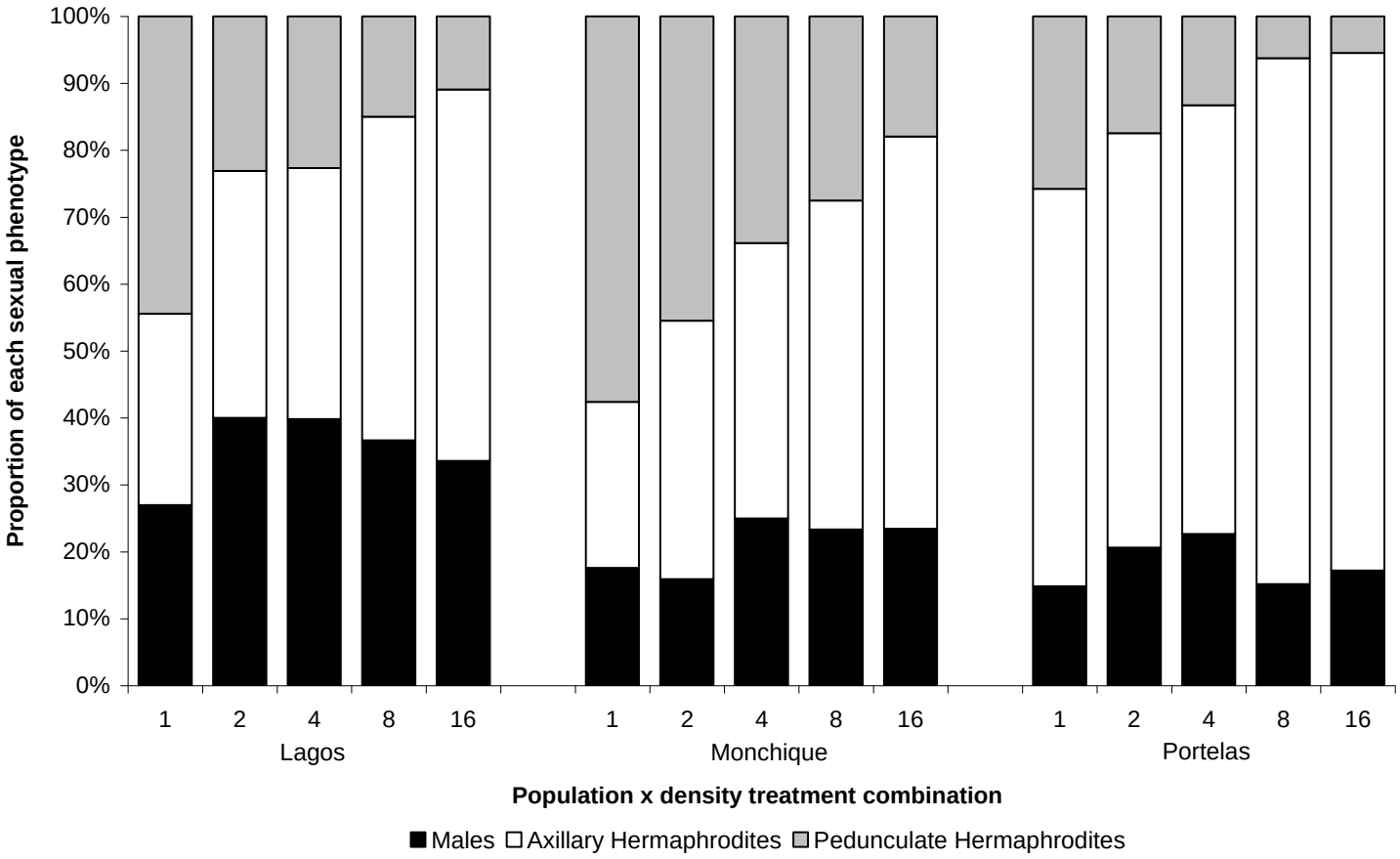


Figure 6.3 The proportion of males, axillary hermaphrodites and pedunculate hermaphrodites scored for each population x density treatment combination in Experiment 2.

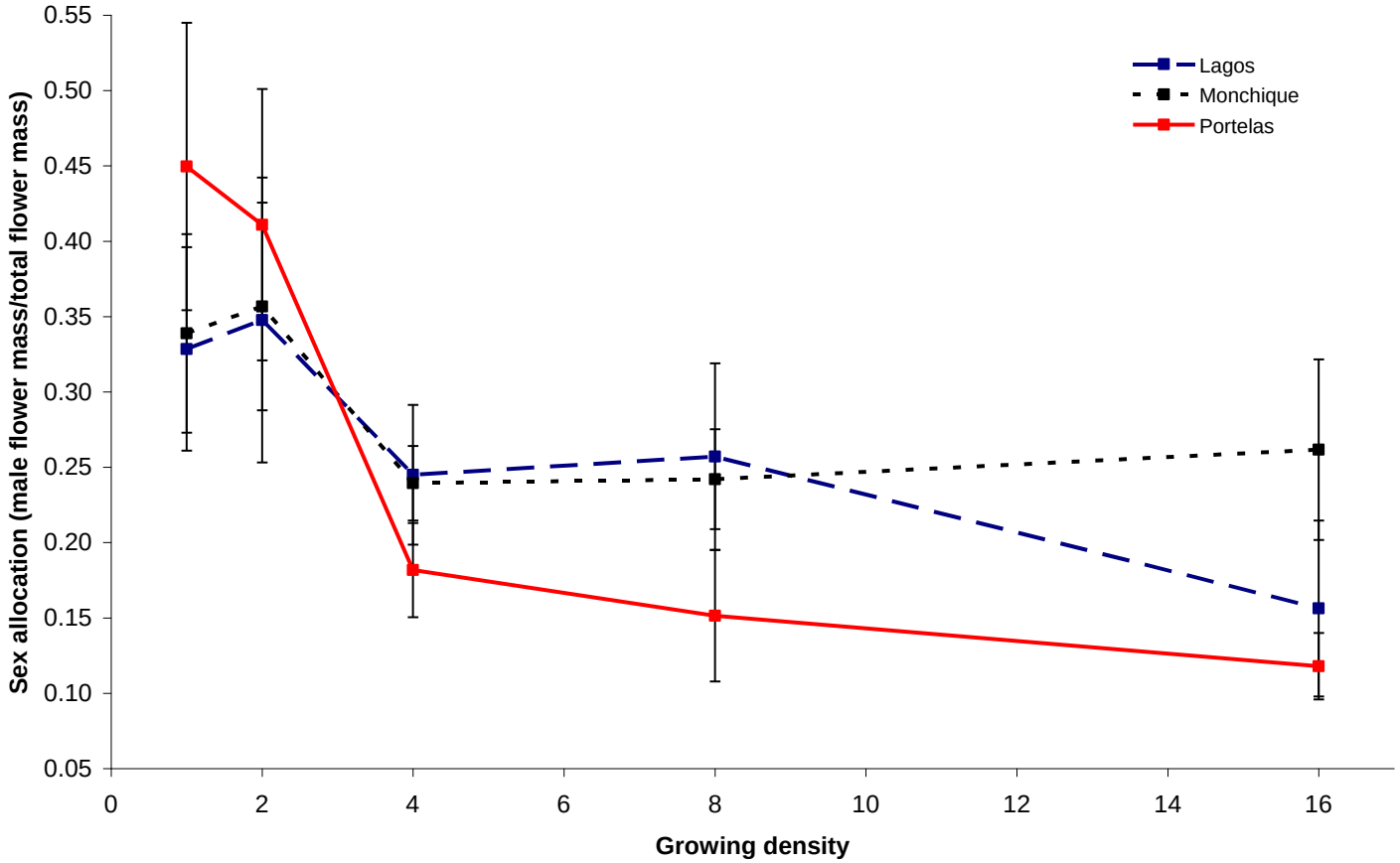


Figure 6.4 Sex allocation (estimated as the proportion of total flower mass allocated to male flowers) of hermaphrodites grown under different density treatments in Experiment 2. Sex allocation became more female-biased with density in all populations, although this effect was weaker in the population from Monchique.

6.4 DISCUSSION

6.4.1 Sex ratio variation with density

The proportion of males produced did not vary significantly with density in any of the six populations tested in Experiments 1 and 2. My study has thus found no evidence for density-dependent gender choice in hexaploid androdioecious *M. annua* in terms of the ratio of males to hermaphrodites produced in populations of the species. In contrast, my study has revealed that the proportion of axillary and pedunculate hermaphrodites is significantly influenced by growing density in a way that also varies among populations. Specifically, the two populations sampled from Morocco and Spain produced very few pedunculate hermaphrodites, unlike the four populations sampled from southern Portugal, in which the proportion of pedunculate hermaphrodites was negatively correlated with plant density and the proportion of axillary hermaphrodites showed a corresponding increase with density. These geographic differences in the floral morphology of hexaploid *M. annua* hermaphrodites are illustrative of the enormous variation in floral morphology in the species (Thomas, 1958; Durand & Durand, 1991), the potential adaptive significance of which is discussed below.

6.4.2 Pedunculate hermaphroditism

In all four of the Portuguese populations studied, a trade-off was detected between the proportions of axillary and pedunculate hermaphrodites produced. As the frequency of males was independent of density in all populations, it would suggest that pedunculate hermaphrodites are 'modified axillary hermaphrodites', i.e. monoecious plants with the capacity to produce peduncles. This differs from the conclusion of Pannell (1997a), who reported a trade-off between males and late

cosexes and subsequently concluded that late cosexes were male individuals which had switched to become hermaphroditic late in their development, i.e. that hermaphroditic individuals which produced male pedunculate inflorescences were 'modified males'. However, from the populations sampled in this study, there is no evidence that males switch gender in response to variation in density. Instead, pedunculate hermaphroditism would appear to represent an alternative floral morphology of hermaphroditic individuals that is more likely to be expressed at low plant density.

Why are hermaphrodites at low density more likely to produce pedunculate inflorescences? One potential reason is that the response reflects resource or size dependence, with peduncles only produced when individuals reach a certain size or are sufficiently well resourced. Total reproductive effort was significantly reduced in pedunculate hermaphrodites compared to axillary hermaphrodites, suggesting that peduncle development is expensive in terms of an individual's resource allocation, and thus peduncles are only produced under resource-rich conditions.

Alternatively, it is possible that the production of peduncles by hermaphrodites at low density has evolved as an adaptive response to variable local mating conditions. *M. annua* is a ruderal coloniser and the mating environments in which individuals develop, in particular the size and density of populations, can vary widely. As the species is wind-pollinated, bearing pollen on erect peduncles would be predicted to increase outcrossing siring success by increasing pollen dispersal distances (Niklas, 1985). Indeed, Eppley and Pannell (2007) found that pollen dispersed from the peduncles of male plants were 1.6 times more likely to sire outcrossed progeny than

that dispersed from axillary hermaphrodites, though it is noted that the peduncles of pedunculate hermaphrodites are smaller than those of males. Nevertheless, a small elevation in pollen dispersal distances would be especially advantageous if conspecifics are sparsely distributed, and thus the production of peduncles by hermaphrodites may have evolved to improve outcrossing siring success in lower-density populations.

In further support of this theory, I found that pedunculate hermaphrodites displayed a significantly more male-biased sex allocation than axillary hermaphrodites. Increased allocation to male function would be predicted to further increase outcrossing siring success in pedunculate, relative to axillary, hermaphrodites. However, as total reproductive effort was reduced in pedunculate relative to axillary hermaphrodites, peduncle development comes at an apparent cost of reducing an individual's overall allocation of resources to reproductive function. At high densities, therefore, where the proximity of conspecific mates means that the production of peduncles is unnecessary for ensuring adequate pollen dispersal, resources are instead allocated towards flower production, chiefly, seed production.

Geographic variation in the occurrence of pedunculate hermaphroditism may also be caused by variations in local mating conditions between populations, with subsequent local adaptation to specific conditions. If the Spanish and Moroccan populations sampled in this study are particularly stable, abundant and large, for example, then the energetic costs of peduncle production may outweigh the benefits of increased outcrossing siring success in these populations. The high male frequency (38%) in the Moroccan population supports the idea of high outcrossing

rates in this population, although male frequency was also relatively high in the Portuguese populations (35% in Lagos), and low in the Spanish population (19%). Alternatively, the observed geographic variation may be attributable to random genetic divergence between populations due to genetic drift and genetic bottlenecks during the lineage's range expansion. Genes for peduncle production appear to segregate in all populations tested in this study (not all hermaphrodites produced peduncles under low density treatments in any of the Portuguese populations) and the very low frequency of pedunculate hermaphrodites in the Moroccan and Spanish populations may thus be due to a lower frequency of 'peduncle-producing genes' in these populations relative to the Portuguese populations. Since hexaploid *M. annua* is hypothesised to have undergone postglacial range expansion (Obbard *et al.*, 2006c), coupled with the metapopulation dynamics of the species (which involves frequent colonisation and extinction events), it is thus possible that drift and founder effects are responsible for geographic variation in the occurrence of pedunculate hermaphroditism in the lineage. Variation in male frequency between androdioecious *M. annua* populations (as likewise observed in this study) has also been partly explained by random genetic divergence between populations, with male frequency thought to be dependent upon the initial frequency and immigration of males and male-determining alleles in a population (Pannell, 1997b; Dorken & Pannell, 2008).

6.4.3 Variation in reproductive effort and sex allocation with density

In addition to the plasticity shown by hermaphrodites in terms of their inflorescence architecture, I found that total, male and female reproductive effort were also significantly influenced by density in males and hermaphrodites of the six

populations tested. The specific effects of density on each of these measures varied between populations; the four populations sampled from southern Portugal responded very similarly to one another, for example, whereas the populations from Morocco and Spain responded more variably, particularly with regard to total reproductive effort. Differences in the effects of density on reproductive effort between populations are further evidence of geographic variation in the sexual lability of androdioecious *M. annua*, which may again be attributable to variations in local mating conditions, or to random genetic divergence between populations, as discussed above.

Most notably, however, the sex allocation of hermaphrodites from all six populations tested responded similarly to density, becoming quantitatively more female-biased with increasing density. This result is in agreement with previous studies of androdioecious *M. annua* (Pannell, 1997b; Dorken & Pannell, 2008; Hesse & Pannell, 2011b) and has been proposed to be a consequence of selection for reproductive assurance in the species. Self-fertile hermaphroditism is hypothesised to have evolved and be maintained in polyploid *M. annua* as a result of selection for reproductive assurance, perhaps during the colonisation of new areas of marginal habitat (see section 5.1 for theories on the evolution of hermaphroditism in polyploid *M. annua*; Pannell, 1997b; Pannell *et al*., 2004). Indeed, there is a widely recognised association between a colonising life history and a capacity for selfing amongst plant species (Baker, 1955; Pannell, 1997d; Pannell & Barrett, 1998; Pannell, 2002). At low density therefore (e.g. when founding a new population), hermaphrodites benefit from an ability to self-fertilise, whereas in high density populations, where males are more likely to be present and the selective pressures

for reproductive assurance are relaxed, hermaphrodites become more female-biased in their sex allocation and enjoy the benefits of increased outcrossing (Pannell, 1997b; 2002).

Alternatively, a change in resource availability as a result of intra-specific competition may explain the female-biased sex allocation of hermaphrodites grown at high density. Hesse & Pannell (2011b) found that androdioecious *M. annua* hermaphrodites grown in poor soils shifted their sex allocation toward increased femaleness, raising the possibility that low resource availability disproportionately limits the production of male as opposed to female flowers. Low nitrogen availability has been implicated in reduced pollen production and disproportionate reductions in male over female reproductive function in other species (e.g. Lau & Stephenson, 1993; Herlihy & Delph, 2009), though in gynodioecious species hermaphroditic sex allocation tends to become more male-biased under resource-limited conditions (e.g. Wolfe & Shmida, 1997; Case & Ashman, 2007). The effects of resource availability on hermaphrodite sex allocation may thus depend on the mating context in which hermaphrodites have evolved, e.g. alongside males or females (i.e. in androdioecious or gynodioecious populations; Hesse & Pannell, 2011b). Alternatively, Hesse & Pannell (2011b) speculate that the mode of pollination may influence the effects of resource availability on sex allocation; the gynodioecious species in which patterns of sex allocation have been studied are all insect pollinated, whereas in a wind pollinated plant such as *M. annua* high resource availability may improve male reproductive success (de Jong & Klinkhamer, 1994; Friedman & Barrett, 2009). For example, under conditions of high resource availability, hermaphrodites would be expected to be taller and may thus improve

pollen grain dispersal (Hesse & Pannell, 2011b). In *M. annua* it is currently unknown whether hermaphrodite size influences outcrossing siring success, but this could be tested experimentally to assess the validity of this hypothesis.

Of course, resource availability may be the cue used by individuals to estimate population density, with changes in quantitative gender arising for alternative adaptive reasons, e.g. in response to the local mating environment, as speculated above. Under this scenario, resource availability would be auto-correlated with sex allocation and the precise causes of, and reasons for, shifts in sex allocation in the species difficult to tease apart. Nonetheless, specific study of these questions would be interesting to elucidate the adaptive significance of the gender plasticity reported in this study.

Plasticity in the sex allocation of hermaphrodites in response to environmental variation has been reported in a number of plant species (Delph & Wolf, 2005), whilst sexual lability more generally is also common amongst plants (Korpelainen, 1998). The sessile nature of plant species is often thought to account for such plasticity; given that plants have little control over the environment they experience, if environmental factors differentially influence fitness as a male or female, an ability to adjust sex expression in response to external stimuli so as to maximise reproductive success is predicted to be favoured over purely genetically controlled sex determination (Charnov & Bull, 1977). In androdioecious *M. annua* the evolutionary significance of sexual lability may be attributable to the species' colonising life history and to variation in local mating conditions, though further

testing of these theories would be worthwhile to improve our understanding of the evolution of ESD in plants.

6.4.4 Conclusions and future work

The results of this study provide strong evidence that hexaploid androdioecious *M. annua* is a highly sexually labile species. Hermaphroditic individuals possess the ability to alter their floral morphology and quantitative gender in response to growing density, with variation in the capacity for such responses between populations across the species' range. The reasons for this plasticity remain unclear, but it may have evolved as an adaptive response to variable local mating environments or resource availability. Future investigation of the adaptive significance of pedunculate hermaphrodites, and testing of whether peduncles do elevate outcrossing siring success, would be particularly interesting. The specific environmental cues that regulate such changes likewise remain unidentified, potentially including soil-borne intraspecific signals, light/nutrient levels, or some other abiotic stimulus, and would also be interesting to elucidate.

The results of Pannell (1997a) nonetheless remain unexplained. Growing density had no effect on the proportion of males produced in any of the six populations tested in this study, whilst hermaphrodites bearing male pedunculate inflorescences were concluded to represent an alternative floral morphology of hermaphroditic individuals, as opposed to males which had switched to become hermaphroditic. It is nevertheless possible that the density-dependent mechanism of sex determination reported by Pannell is unique to populations in and around Seville. Despite no evidence of density influencing male frequency in this study, populations did

respond differently to density in terms of reproductive effort and the inflorescence architecture of hermaphrodites, indicating geographic variation in the capacity for density-dependent gender choice exists in the species. It would therefore be worthwhile to obtain seed from new populations of androdioecious *M. annua* around Seville in order to test for the presence of density-dependent sex determination in this locality. Nonetheless, based on the results of this investigation, I conclude that the development of hexaploid *M. annua* individuals as either male or hermaphrodite is determined purely genetically by a single-locus mechanism, males arising by the inheritance of a dominant allele at this locus, with hermaphrodites homozygous recessive, as outlined in previous chapters of this thesis (see Chapters 2 & 5).

7. GENERAL DISCUSSION

7.1 INTRODUCTION

This thesis set out to investigate the determination of sex in the plant species *Mercurialis annua*. Sex determination is a fundamental biological process and a key fitness component of sexually reproducing organisms, as it regulates the allocation of resources to male and female reproductive function to maximise reproductive success. Sex-determining mechanisms may also have implications for a species' genome, precipitating the evolution of non-recombining, highly degenerate sex chromosomes, and a species' physiology, through the development of secondary sexual dimorphisms. *M. annua* has been a model system for the study of sex determination in plants for almost a century (Yampolsky, 1919; 1957; Gabe, 1939; Durand, 1963; Louis, 1989; Durand & Durand, 1991; Pannell, 1997a; Khadka *et al*., 2002; 2005), but numerous questions surrounding gender determination in the species remained unanswered; I sought to address some of these in this study. Specifically, this thesis examined: i) the mechanism of sex determination operating in dioecious *M. annua* (Chapter 2); ii) the conservation of sex determination amongst lineages of the annual mercury clade (Chapters 3 & 5); iii) the evolution, and implications for genetic diversity, of suppressed recombination around sex-determining loci in dioecious *M. annua* (Chapters 3 & 4); iv) the effects of polyploidisation and hybridisation on sex determination in the annual mercury clade (Chapter 5); and v) the presence of environmental sex determination (ESD) and sexual lability in androdioecious *M. annua* (Chapter 6).

Below, I briefly summarise the key findings of this study and discuss these results in a broader context, comparing what has been learnt in *M. annua* to what is known about sex determination in other species. I also discuss the limitations of this study, outline further work that can be undertaken to build on this thesis, and consider the contribution that study of sex determination in *M. annua* can make to our understanding of the evolution of sex-determining mechanisms more generally.

7.2 SEX DETERMINATION IN *M. ANNUA* AND THE ANNUAL MERCURIES

The results of my investigations in Chapters 2, 5 and 6 point towards a conserved mechanism of single-locus genetic sex determination across the annual mercury clade. The exclusive co-segregation of the DNA marker *OPB01-1562* with male sex in controlled crosses of dioecious *M. annua* (Chapter 2) provides strong evidence for a single-locus mechanism of sex determination in this lineage, with *OPB01-1562* putatively linked to a dominant male-determining allele. These results contradict a widely cited three-locus model that has also been purported to explain sex determination in dioecious *M. annua*, but under which no allele is exclusively linked to a single sex (Louis, 1989). Unlike the multi-locus model, a single-locus model is also in accordance with theoretical predictions for the development of sex-determining mechanisms following the evolution of dioecy in plants; gender is predicted to be controlled by a single, rarely recombining chromosomal region following the evolution of dioecy from hermaphroditism in order to prevent the break up of gene combinations conferring complementary sterility and functionality in either male or female reproductive function (Charlesworth & Charlesworth, 1978a; 1980; Rice, 1984; 1987a; Charlesworth, 2002). A single-locus mechanism of sex determination in dioecious *M. annua* thus makes the species comparable with

numerous other dioecious species in which gender is also purported to be controlled by single-locus systems, including *Asparagus officinalis* (Loptien, 1979; Telgmann-Rauber *et al.*, 2007), *Sagittaria latifolia* (Dorken & Barrett, 2004) and *Spinacia oleracea* (Khattak *et al.*, 2006; Lan *et al.*, 2006).

The rejection of the three-locus model also raises interesting questions regarding whether multi-locus systems of sex determination are able to evolve and be maintained in dioecious plants? As far as I am aware, dioecious *M. annua* is the only dioecious plant species in which such a mechanism has been proposed. Multi-locus or polygenic sex determination may be an intermediate state in the evolution of a species' sex-determining mechanism, for example, when mutations controlling male and female reproductive function initially arise, but are thought to be evolutionarily unstable and vulnerable to displacement by 'monogenic' sex determination (Bull, 1981; 1983; Rice, 1986; Uller *et al.*, 2007). Were a single major sex-determining gene to arise which pleiotropically increased fitness itself, or was linked to a selectively advantageous gene, for example, it would be predicted to displace a polygenic sex-determining mechanism (Rice, 1986). In dioecious plants, the prediction that male- and female-sterility mutations which become established during the evolution of dioecy must be genetically linked would likewise preclude the development of multi-locus systems of sex determination. It is worth emphasising, of course, that these predictions apply only to dioecious species, where gender is fully separated between male and female individuals; polygenic systems of sex determination have been proposed for the gynodioecious species *Plantago coronopus*, *Silene vulgaris* and *Thymus vulgaris* (Ehlers *et al.*, 2005), where the exclusive maternal inheritance of mitochondrial sterility genes enables these genes

to be transmitted through both sexual morphs, whilst in functionally hermaphroditic species gender may be controlled by a complex mixture of many loci of small effect and environmental factors. Nevertheless, further study of sex determination in the few animal species in which polygenic mechanisms have been proposed (e.g. the fish species *Xiphophorus helleri* and *Dicentrarchus labrax*; Kosswig, 1964; Vandeputte *et al.*, 2007) would be valuable for examining the evolution and maintenance of these rare systems.

Following the elucidation of single-locus sex determination in dioecious *M. annua*, evidence from hybridisations between various annual mercury lineages indicates this system to be conserved across the annual mercury clade (Chapter 5). All hybridisations involving a male plant yielded male progeny in a ratio of 1:1 with either females or hermaphrodites (except in the case of the diploid *M. annua* x *M. huetii* hybridisation, where there was a significant departure from 1:1; see section 5.4.2), indicating that male-determining alleles from dioecious *M. annua*, androdioecious *M. annua* and *M. huetii* are capable of producing a male phenotype in the genetic background of all other annual mercury lineages. Despite *OPB01-1562* not being conserved as a sex-linked marker in other lineages (Chapter 3), these results indicate a conserved basis for sex determination across the clade. From the results obtained in this study, however, one is unable to distinguish between whether there exists a single conserved male-determining gene across the clade, or whether different genes at different loci give rise to males in each lineage, with a conserved downstream mechanism of sex determination across the clade.

Since no crosses involving polyploid *M. annua* yielded hybrids with a female phenotype (progeny were exclusively male or hermaphroditic if polyploid *M. annua* were used in crosses), it would imply that polyploid *M. annua* possesses genes for producing axillary male flowers and restoring a monoecious phenotype which are dominant to genes controlling the development of females in dioecious lineages. However, such monoecious hybrids produced very few male flowers, suggesting that male flower production is suppressed to some extent in the background of dioecious lineages by female-determining genes. Based on these findings, I propose a simple genetic model to explain sex determination across the annual mercury clade. Maleness is conferred by the inheritance of a dominant allele at a single locus in all lineages. In the absence of this allele, individuals develop as females or monoecious hermaphrodites depending on the inheritance of alleles controlling male flower development at other loci potentially fixed elsewhere in the genome, and not necessarily at the same locus as the male-determining allele; dioecious lineages suppress male flower development, giving rise to females, whilst monoecious lineages promote axillary staminate flower development. In hermaphroditic hybrids of dioecious and monoecious lineages, axillary male flower production is intermediate between that of the two systems, i.e. it is partially suppressed by alleles from the dioecious system, but a small number of flowers are produced, presumably by alleles of the monoecious system.

In other species and genera with both monoecious and dioecious populations, crosses between individuals of different sexual system have yielded sex ratios both similar and very different to those reported in *M. annua* in this study. For example, *Sagittaria latifolia* (Dorken & Barrett, 2004) and *Ecballium elaterium* (Galán, 1951)

have both been inferred to possess similar mechanisms of single-locus sex determination with male heterogamety as that proposed in *M. annua*, with male-determining alleles being interpreted as dominant to monoecious-determining alleles, which are in turn dominant to female-determining alleles. In contrast, crosses between androdioecious and dioecious *Datisca* species (Wolf *et al.*, 2001), and between monoecious and dioecious *Bryonia* species (Correns, 1903; 1907), yielded only male and female offspring, from which it was concluded that hermaphroditism is recessive to male- and female-determining alleles. Differences in sex-determining mechanisms between species are most likely the result of differences in evolutionary history, particularly the origin of monoecy and dioecy, between lineages. For example, dioecy is hypothesised to have evolved from monoecy in *Sagittaria latifolia* via the gynodioecy pathway, with the initial establishment of recessive male-sterility mutations giving rise to intermediate populations containing females and monoecious hermaphrodites (see section 2.1; Charlesworth & Charlesworth, 1978a; Dorken & Barrett, 2004). Selection for dominant female-sterility mutations in hermaphrodites is then thought to have led to the development of dioecious populations, with a sex-determining system of male heterogamety, in which female-determining alleles are also recessive to monoecious-determining alleles, thus produced (Dorken & Barrett, 2004). In contrast, in *Datisca glomerata*, androdioecy has been proposed to have evolved from dioecy following the spread of a recessive mutation that allowed females to produce pollen, hence hermaphroditism is recessive to femaleness in crosses between *D. glomerata* and its dioecious sister species, *D. cannabina* (Wolf *et al.*, 2001). In the *M. annua* species complex, the evolution of monoecy is hypothesised to have been precipitated by polyploidisation; all polyploid populations are monoecious or androdioecious whilst

diploid populations are exclusively dioecious, with dioecy also believed to be ancestral in the annual mercury clade and possibly in the genus *Mercurialis* (Durand, 1963; Durand & Durand, 1992; Krähenbühl *et al.*, 2002; Obbard *et al.*, 2006b).

Polyploidisation may have led to the development of functional hermaphroditism in *M. annua* by either direct disruption of the sex-determining mechanism of the dioecious system, or as a result of selection for functional hermaphroditism in neo-polyploids (Westergaard, 1958; Pannell *et al*., 2004). In this thesis, artificial reconstruction of autotetraploid *M. annua* by colchicine-induced polyploidisation of diploid *M. annua* did not produce any monoecious neo-polyploids, suggesting that genome duplication alone may be insufficient for the production of a monoecious phenotype and that selection for functional hermaphroditism may have been involved in the evolution of monoecy in the species (Chapter 5). This finding is consistent with the proposed model for sex determination in the annual mercuries, which suggests that alleles promoting axillary male flower production are necessary for the production of a monoecious phenotype, but that these alleles are absent in the genotypes of diploid dioecious *M. annua* and thus must have been selected for in neo-polyploids. However, the results presented in this thesis differ from those of Durand (1963), who observed monoecious individuals in the progeny of artificial *M. annua* autotetraploids. In *Empetrum* (Richards, 1997) and *Isotoma* (McComb, 1968), rare examples of plant species in which functional hermaphroditism is also reported to have evolved following polyploidisation, the disruption of sex determination has been suggested to have caused the shift in mating system, rather than selection for cosexuality. It is therefore possible that a larger sample of neo-tetraploids needs to be examined in order to observe the disruption of sex

determination and the segregation of a monoecious phenotype (only 14 neo-tetraploids were produced by this study) and further study of this subject is hence proposed in Chapter 5.

With regard to the origin of androdioecy in hexaploid *M. annua*, this rare sexual system may have been selected from dioecy (Pannell, 2001), as has been suggested in *D. glomerata* and the small number of other plant species in which it has been reported, including *Spinifex littoreus* (Connor, 1996), *Schizopepon bryoniaefolius* (Akimoto *et al.*, 1999) and *Castilla elastica* (Sakai, 2001). Alternatively, androdioecy is thought more likely to have evolved as a direct result of hybridisation, with genes for a fully functional male phenotype inherited from *M. huetii*, the putative paternal progenitor of this allohexaploid (Obbard *et al*., 2006b). This hypothesis is supported by two lines of evidence in this thesis: first, the fact that male-determining alleles of annual mercury lineages appear functional in the genetic background of all other lineages, and hence hybridisations between male *M. huetii* and tetraploid *M. annua* (the putative maternal progenitor of hexaploid *M. annua*) yielded male progeny (Chapter 5); and second, that the putatively male-linked marker *OPB01-1562* of dioecious *M. annua* does not amplify in hexaploid males or *M. huetii* males, suggesting that the male-determining genes of androdioecious *M. annua* have not been inherited from the dioecious system (Chapter 3). However, in order to provide explicit evidence for a hybrid origin of androdioecy in hexaploid *M. annua*, I suggest that the identification of homologous sex-linked genes between male *M. huetii* and hexaploid *M. annua* is necessary. The identification of sex-linked genes amongst annual mercuries more widely would also be an extremely valuable tool for further assessing the conservation of sex determination across the

annual mercury clade. This goal may be achieved by building on the segregation analysis of gene transcripts in dioecious *M. annua* described in Chapter 3, as identification of active sex-linked genes may enable elucidation of those conserved across the annual mercury clade, or even across the *Mercurialis* genus.

In addition to a conserved mechanism of genetic sex determination being identified amongst annual mercuries, the results presented in Chapter 6 of this thesis indicate there is no evidence for shifts in the sex ratios of androdioecious *M. annua* populations in response to growing density. Pannell (1997a) reported gender in androdioecious populations to be controlled by a mixed genetic-environmental system of sex determination, with males determined by the inheritance of a dominant allele at a single locus (corroborating the findings described above) and male frequency also influenced by growing density. However, I find no evidence of growing density influencing male frequency in androdioecious populations. I therefore conclude that the development of hexaploid *M. annua* individuals as either a male or monoecious hermaphrodite is controlled by a simple single-locus genetic mechanism, as outlined above.

That said, a degree of sexual lability was detected amongst hermaphrodites of androdioecious *M. annua* populations (Chapter 6). The proportion of pedunculate hermaphrodites produced (hermaphroditic plants which bore some male flowers on pedunculate inflorescences) was found to be negatively correlated with growing density, whilst the proportion of axillary hermaphrodites produced (which bore all male and female flowers in leaf axils) showed a corresponding increase with density. Pedunculate hermaphrodites were also found to display a more male-biased sex

allocation than axillary hermaphrodites, consistent with the hypothesis that pedunculate hermaphroditism evolved to improve outcrossing siring success in lower-density populations. Furthermore, a correlation between the sex allocation of both hermaphrodite types and growing density was also detected, individuals becoming quantitatively more female-biased with increasing density. This result is in agreement with previous work on the species (Pannell, 1997b; Dorken & Pannell, 2008; Hesse & Pannell, 2011b), and is believed to be a consequence of either selection for reproductive assurance (with elevated male allocation at low densities for increased selfing, but elevated female allocation at high densities for increased outcrossing), or changes in resource availability. Sexual lability is well documented in plant species (reviewed in Korpelainen, 1998), and may be attributable to the sessile nature of plants; if environmental factors differentially influence male and female fitness and the environment is heterogeneous with respect to such factors, it is advantageous for individuals to be able to alter the allocation of resources to male and female reproductive function, and hence ESD in some form is predicted to be favoured (Charnov & Bull, 1977). In androdioecious *M. annua*, such a capacity may be favoured as a result of the species' colonising life history. For example, the ability to alter sex allocation in response to the local mating environment, or to changes in resource availability as a result of intra-specific competition, may be advantageous in a species where individuals have little control over both the biotic and abiotic environment they will experience (Pannell, 1997b; Hesse & Pannell, 2011b). Nevertheless, changes in the proportion of pedunculate hermaphrodites with density, and associated trade-offs in sex allocation, have not been previously estimated in androdioecious *M. annua* and suggest a high level of plasticity in floral development of the species. Furthermore, the ability of hexaploid hermaphrodites to

produce male peduncles suggests that they retain genes and genetic pathways for producing this inflorescence which are activated only under certain environmental conditions. Future study investigating the fitness benefits of peduncles in androdioecious *M. annua* hermaphrodites would be extremely interesting. Does, for example, the presence of peduncles increase outcrossing success at low density? At first glance this appears the most likely explanation for the increased proportion of pedunculate hermaphrodites at low density and their male-biased sex allocation relative to axillary hermaphrodites, but future study would be worthwhile to address this question.

7.3 SEX CHROMOSOME EVOLUTION IN DIOECIOUS *M. ANNUA*

In Chapter 3, my search for new sex-linked genes in dioecious *M. annua* by segregation analysis of sequenced gene transcripts identified numerous putatively sex-linked genes, but further examination found that the sex-linkage of these genes was not maintained in wild populations. This result led to the interesting conclusion that the species either lacks a non-recombining region, or that any non-recombining region around sex-determining loci is relatively small (although the exclusive co-segregation of *OPB01-1562* with male sex does indicate some degree of recombination suppression). Given that dioecy is assumed to be the ancestral condition in the annual mercury clade, and possibly in the *Mercurialis* genus (Durand & Durand, 1992; Krähenbühl *et al.*, 2002; Obbard *et al.*, 2006b), it is perhaps surprising that recombination suppression appears not to have expanded significantly in the sex chromosome pair of dioecious *M. annua*. Nonetheless, this scenario appears to be the rule rather than the exception amongst dioecious plant species; sex-determining mechanisms consisting of a single, or several very tightly

linked, loci located in a small non-recombining region (although in some cases recombination is not even fully suppressed, e.g. *Fragaria virginiana*; Spigler *et al.*, 2008) have been reported in several dioecious plant species from a range of angiosperm families, including *Asparagus officinalis* (Asparagaceae; Telgmann-Rauber *et al.*, 2007), *Spinacia oleracea* (Chenopodiaceae; Khattak *et al.*, 2006; Lan *et al*., 2006) and *Sagittaria latifolia* (Alismataceae; Dorken & Barrett, 2004). In contrast, heteromorphic sex chromosomes have been identified in just four angiosperm families (Ming *et al.*, 2011): Cannabaceae; Caryophyllaceae (all being *Silene* sp.; Westergaard, 1958; Nicolas *et al.*, 2005); Cucurbitaceae; and Polygonaceae (all being *Rumex* sp.; Ono, 1935; Navajas-Pérez *et al.*, 2005). The reasons for this rarity are unclear; it is often attributed to the recent evolution of dioecy and high turnover in sexual systems amongst plant species, meaning the sex-determining mechanisms of dioecious species are generally quite young. Nevertheless, heteromorphic sex chromosomes have evolved in *S. latifolia* over the last 5 to 10 million years (Bergero *et al.*, 2007), and tentative estimates suggest this is a timescale not dissimilar to the evolution of dioecy in *Mercurialis*.

Another reason purporting to explain the lack of extensive recombination suppression around the sex-determining loci of many dioecious plants is a lack of selection for sexual dimorphism in plant species. It has been suggested that lower selection for sexual dimorphism in plants compared to animals reduces the fitness advantages of linking sexually antagonistic mutations to the sex-determining locus (Charlesworth, 2008; Charlesworth & Mank, 2010). Small regions of recombination suppression are subsequently maintained in many plant systems. In Chapter 4, the nucleotide diversity levels of partially sex-linked loci were estimated in dioecious *M.*

annua and found not to be significantly different to those of putatively autosomal loci (the diversity of *OPB01-1562*, a putatively Y-linked marker, was potentially lower than autosomal loci, however, as theory predicts). If sexually antagonistic alleles are accumulating on the putative Y chromosome of the species, then one might expect levels of diversity to be elevated between X and Y chromosomes, but such a pattern was not detected in this study. However, sexual dimorphism is apparent in many plant species (Geber *et al.*, 1999), including both *M. annua* and *S. latifolia*, and a recent study has indicated that sex-linked quantitative trait loci contribute heavily to sexual dimorphism in *F. virginiana* (Spigler *et al.*, 2011). Why *S. latifolia* and not *M. annua* should develop heteromorphic sex chromosomes thus remains unexplained.

7.4 *M. ANNUA* AS A MODEL SPECIES

The work presented in this thesis has established the general mechanism of sex determination present in *M. annua* and raised several questions with regard to: i) the evolution and maintenance of sex determination across the annual mercury clade; and ii) the evolution of sex chromosomes in dioecious *M. annua*. Given the favourable life history of the species for greenhouse cultivation (it is small, fast-growing and very easy to maintain), allied to the interesting variation it displays in terms of its sexual system and ploidy levels, *M. annua* is ideally suited to be a model organism for the study of sex determination and sex chromosome evolution in dioecious plants. The sex-determining mechanism of the dioecious lineage has already been studied for almost a century (Yampolsky, 1919; 1957; Gabe, 1939; Durand, 1963; Louis, 1989; Durand & Durand, 1991; Pannell, 1997a; Khadka *et al.*, 2002; 2005), and the species has been more recently used for study of sexual system

evolution (Pannell, 1995; 1997c; Pannell *et al.*, 2004; Obbard *et al.*, 2006b), sex allocation strategies (Pannell, 1997b; Hesse & Pannell, 2011b; Sánchez-Vilas & Pannell, 2012), polyploid evolution (Obbard *et al.*, 2006b) and population dynamics (Buggs & Pannell, 2006; Obbard *et al.*, 2006c). However, I believe it also has great potential as a model organism for studying questions regarding the evolution and maintenance of sex determination and sex chromosomes.

The work presented in this thesis potentially represents the starting point for an in-depth study of the evolution of sex determination and sex chromosomes in *M. annua*. Identifying new fully sex-linked genes in dioecious *M. annua* by undertaking a second generation of crosses from the segregation analysis of sequenced gene transcripts (Chapter 3) would be a logical starting point. The identification of new sex-linked genes would be extremely beneficial for the study of several aspects of sex determination in the species, including: the conservation of specific sex-determining genes across the annual mercury clade and the genus *Mercurialis*; divergence between putatively X- and Y-linked genes; potential degeneration of Y-linked genes; and the full extent of recombination suppression in dioecious *M. annua*. Furthermore, identification of partially sex-linked genes (those genes located on the sex chromosome pair but in regions in which recombination still occurs) would permit further examination of patterns of genetic diversity in different genomic regions, as well as a potential assessment of whether sexually antagonistic genes are accumulating on the sex chromosome pair as theory predicts. Elucidation of the precise homology of the sex-determining system of androdioecious *M. annua* and other annual mercury lineages may also enable the origin of this rare mating system to be clarified and facilitate examination of the role

polyploidisation has played in the evolution of functional hermaphroditism in the species. Furthermore, the detection of family sex ratio variation amongst half-sib and full-sib seed families of dioecious *M. annua* (Chapter 2) also permits study of this phenomenon in the species, whilst the sexual lability reported in androdioecious *M. annua* populations likewise has the potential for further study to examine the precise cues and adaptive reasons for this plasticity.

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9. APPENDICES

9.1 *MERCURIALIS* POPULATIONS FROM WHICH SEEDS WERE USED FOR EXPERIMENTAL WORK PRESENTED IN THIS THESIS

Table 9.1 The origin of *Mercurialis* populations from which seeds were used for experimental work presented in this thesis. 'Section' refers to the section(s) of the thesis in which seeds from each population were used.

Code	Location	Country	Latitude	Longitude	Sexual system	Section(s)
19a	Pradell de la Teixeta	Spain	41.15747N	0.87758E	Dioecious	2.2.1
31a	C246, nr. Tarragona	Spain	41.197778N	1.601944E	Dioecious	2.2.1
330	A7, nr. Cambrils	Spain	41.05735N	0.99443E	Dioecious	2.2.1
331	Cambrils	Spain	41.07948N	1.06297E	Dioecious	2.2.1
34a	B211, nr. Sant Pere de Ribes	Spain	41.26090N	1.75946E	Dioecious	2.2.1
37a	N340, nr. L'Arboç	Spain	41.29475N	1.62561E	Dioecious	2.2.1
40a	N240, nr. Tarragona	Spain	41.21417N	1.24917E	Dioecious	2.2.1
41a	Sitges	Spain	41.23306N	1.79250E	Dioecious	2.2.1
584b	N632, nr. Ribadesella	Spain	43.44947N	-5.05075E	Dioecious	2.2.1
AR	Andover Road, Winchester	UK	51.06813N	-1.31784E	Dioecious	2.2.1
BS	Bond Street, Southampton	UK	50.91119N	-1.38406E	Dioecious	2.2.1
CMa	Cripley Meadow, Oxford	UK	51.76046N	-1.27355E	Dioecious	2.2.1
PS	Princes Street, Southampton	UK	50.91191N	-1.38581E	Dioecious	2.2.1
TB	Tel Aviv Botanical Garden	Israel	32.11377N	34.80883E	Dioecious	2.2.1
TE	Tel Aviv Educational TV	Israel	32.11493N	34.80759E	Dioecious	2.2.1
J	Jerusalem Great Synagogue	Israel	31.77581N	35.21694E	Dioecious	2.2.1, 3.2.3, 4.2.1
RB	Rehovot Bialer 6 Street	Israel	31.87829N	34.81743E	Dioecious	2.2.1, 4.2.1
232a	Sestri Levante	Italy	44.27127N	9.42143E	Dioecious	2.2.2
98a	HaGoshrim	Israel	33.22083N	35.62361E	Dioecious	2.2.2
668b	Tarragona	Spain	41.18560N	1.24247E	Dioecious	2.2.2, 3.2.1, 3.2.3, 4.2.1

Table 9.1 (continued)

Code	Location	Country	Latitude	Longitude	Sexual system	Section(s)
200a	Tenerife	Spain	28.3939N	-16.6361E	*canariensis* (dioecious)	3.2.1
1020a	nr. Dar-ed-Dou	Morocco	33.1324N	-8.6131E	Monoecious	3.2.1, 5.2.1
1044a	Ksar el-Kebir	Morocco	35.0163N	-5.9148E	Androdioecious	3.2.1, 5.2.1
719a	Portbou	Spain	42.43448N	3.16716E	*huetii* (dioecious)	3.2.1, 5.2.1
500a	Castelldefels sud	Spain	41.27204N	1.96954E	Dioecious	3.2.3, 5.2.1, 5.2.3
78a	Plymouth	UK	50.37639N	-4.11611E	Dioecious	3.2.3, 4.2.1
80a	Izmit	Turkey	40.78N	29.92E	Dioecious	3.2.3, 4.2.1
68a	Cephalonia	Greece	38.25N	20.58E	Dioecious	3.2.3, 4.2.1
228a	Paestum	Italy	40.419N	15.004E	Dioecious	3.2.3, 4.2.1
1562a	Mont de Marsan	France	43.88421N	-0.49576E	Dioecious	4.2.1
579a	Bonastre	Spain	41.23810N	1.42765E	Dioecious	4.2.1
591a	Espasante	Spain	43.71207N	-7.80015E	Dioecious	4.2.1
65a	Saegewerk, nr. Getzersdorf	Austria	48.32N	15.72E	Dioecious	4.2.1
70a	Corbais	Belgium	50.65444N	4.66528E	Dioecious	4.2.1
FT1	Asos Peninsula	Greece	38.38111N	20.53083E	Dioecious	4.2.1
1053a	Trebujena	Spain	36.82570N	-6.21640E	Androdioecious	6.2.1
1525a	Monchique	Portugal	37.305N	-8.564E	Androdioecious	6.2.1
MOR1	Ksar el-Kebir	Morocco	35.003N	-5.911E	Androdioecious	6.2.1
1520a	Portelas	Portugal	37.1242N	-8.6948E	Androdioecious	6.2.2
1521a	Lagos	Portugal	37.107N	-8.677E	Androdioecious	6.2.2
1522a	Monchique	Portugal	37.305N	-8.564E	Androdioecious	6.2.2

9.2 PHOTOGRAPHS OF *MERCURIALIS* LINEAGES AND HYBRIDS STUDIED IN CHAPTER 5

Figure 9.1 Photograph of diploid *Mercurialis annua* (left), *M. huetii* (right) and a diploid *M. annua* x *M. huetii* hybrid (centre) studied as part of the comparative morphological analysis presented in Chapter 5. Note the intermediate morphology of the hybrid relative to its progenitors.

Figure 9.2 Photograph of *M. huetii* (left), tetraploid *M. annua* (right) and a *M. huetii* x tetraploid *M. annua* hybrid (centre) studied as part of the comparative morphological analysis presented in Chapter 5. Note the greater morphological similarity of the hybrid to tetraploid *M. annua* than to *M. huetii*.

Figure 9.3 Photograph of diploid *M. annua* (left), tetraploid *M. annua* (right) and a diploid *M. annua* x tetraploid *M. annua* hybrid (centre) studied as part of the comparative morphological analysis presented in Chapter 5.

Figure 9.4 Photograph of diploid *M. annua* (left), male hexaploid *M. annua* (right) and a diploid *M. annua* x male hexaploid *M. annua* male hybrid (centre) studied as part of the comparative morphological analysis presented in Chapter 5. Note the increased number of pedunculate inflorescences (and subsequent increased reproductive effort) of the hybrid relative to the male hexaploid plant.

Figure 9.5 Fruits of hybrid (left) and non-hybrid (right) *M. annua* individuals sampled from plants studied in Chapter 5. Hybrids had exceptionally low female fertility and the majority of fruits were unfertilised, remaining small with long feathery stigmas, as shown above. In comparison, non-hybrid progeny had significantly greater female fertility and fruits developed normally. These differences in fruit morphology enabled visual identification of hybrid and non-hybrid progeny.