Molecular and morphological description of *Haemoproteus (Parahaemoproteus) bukaka* (*species nova)*, a haemosporidian associated with the strictly Australo-Papuan host Subfamily Cracticinae.

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Running title: Haemosporidians in cracticines

Word count: Total = 6411

Abstract = 244

Main Text = 4671

Acknowledgments = 157

References = 1249

Number of references = 46

Number of tables = 2 (including Appendix)

Number of figures = 3 (including Appendix)

Abstract.

Linking morphological studies with molecular phylogeny is important to understanding cryptic speciation and the evolution of host-parasite relationships. Haemosporidian parasites of this Australo-Papuan bird family Artamidae are relatively unstudied with only one parasite species from the subfamily Cracticinae having been described, and this was based solely on morphological description. This is despite many Cracticinae species being easily observed and abundant over large ranges and in close proximity to human populations. We used morphological and molecular methods to describe a new *Haemoproteus* species (*H. bukaka* sp. nov.) from an endemic Butcherbird host (*Cracticus louisiadensis*) in a relatively unstudied insular area of high avian endemism (Papua New Guinea's Louisiade Archipelago). Phylogenetic reconstructions using parasite cyt-*b* gene sequences placed the proposed *Haemoproteus bukaka* sp. nov. close to other host-specialist *Haemoproteus* species that infect meliphagid honeyeater hosts in the region, e.g. *H. ptilotis*. Distinct morphological characters of this haemosporidian include macrogametocytes with characteristic large vacuoles opposing a subterminal nucleus on the host cell envelope. Among 27 sampled individuals, prevalence of *H. bukaka* sp.nov. was high (74% infection rate) but strongly variable across 4 islands in the archipelago (ranging from 0-100% prevalence). Parasitaemia levels were low across all infected individuals (0.1-0.6%). We suspect host density may play a role in maintaining high prevalence given the close proximity and similar physical environments across islands. The findings are discussed in the context of the host genus *Cracticus*, and theory relating to parasite-host evolution and its conservation implications in Papua New Guinea.

Keywords: Papua New Guinea; Haemoproteus; parasites; endemic birds; islands; Artamidae.

1. Introduction.

The Australo-Papuan region represents one of the least sampled and most underrepresented global regions in the study of avian haemosporidians to date (Clark et al. 2014). However, the Australo-Papuan region supports evolutionary distinct avifauna that would be expected to host equally distinct haemosporidian diversity. This is particularly likely with *Haemoproteus* species, which tend to show a greater level of avian host specialisation than *Plasmodium* species (Clark et al. 2014; Zhang et al. 2014; Reeves et al. 2015). To date, only one *Haemoproteus* species in Australia has been described using both molecular and morphological techniques (*H. ptilotis*; Clark et al. 2015). To increase global knowledge of the diversity of these parasites and the evolution of host-parasite relationships, further efforts combining both techniques are required from poorly studied biogeographic regions (Adlard et al. 2004; Matta et al. 2014).

Avian haemosporidians are intra-erythrocytic parasites that infect a diverse array of avian hosts worldwide and are transmitted by biting dipteran vectors (Valkiūnas 2005; Atkinson et al. 2009). Microscopic methods have traditionally been used to describe their morphological characteristics and have resulted in the description of more than 206 species (Valkiūnas 2005). However, the application of molecular Polymerase Chain Reaction (PCR) techniques largely targeting portions of the haemosporidian Cytochrome-*b* gene (cyt-*b*) have rapidly expanded the global knowledge-base of these parasites and revealed potentially hidden diversity (Atkinson 2009; Bensch et al. 2009). There are now over 2 000 genetic lineages of avian haemosporidian parasites (*Leucocytozoon*, *Plasmodium* and *Haemoproteus*; Order Haemosporida) registered with the global MalAvi Database (Bensch 2015). However, determining the diversity of these parasites using the species concept is hampered by the large knowledge gaps in complete life-cycles and the lack of combined molecular and morphological descriptions (Clark et al. 2014; Outlaw and Ricklefs 2014).

The avian Subfamily Cracticinae (genera *Cracticus*, *Strepera* and *Peltops*) has members with endemicity restricted to Australia and New Guinea (Kearns et al. 2013). The most speciose and prominent amongst these are the Butcherbirds (*Cracticus spp*.). Members of this genus are not cryptic in behaviour, often abundant and widely distributed. *Cracticus* species occur from the cool, temperate island of Tasmania in the south of Australia to the tropical islands of New Guinea in the north. There are conservatively eight species recognised in the genus

Cracticus (Christidis and Boles 2008; Kearns et al. 2013). Three of these are found solely in Australia (*C. nigrogularis, C. mentalis* and *C. torquatus*), two in both Australia and New Guinea (*C. tibicen* and *C. quoyi*), and two restricted to New Guinea (*C. cassicus and C. louisiadensis;* Kearns et al. 2013). Most are widespread in open forest and forest edge/disturbed habitat, except for those found typically in dense forest, the Black Butcherbird (*C. quoyi*) of northern Australia and New Guinea, and the Hooded and Tagula Butcherbirds of New Guinea (*C. cassicus* and *C. louisiadensis*, respectively).

Investigations of the haemosporidian parasites of *Cracticus* species are limited. *Leucocytozoon artamidis* is the sole haemosporidian species described that infects birds in this family in Australia (F. Artamidae; Peirce et al. 2005). These infections were characterised using light microscopy to identify parasite morphological characters. Identified *Cracticus* hosts were *C. torquatus, C. nigrogularis, C. tibicen* and the closely related Pied Currawong in southeast Queensland, Australia (*Strepera graculina*; Peirce et al. 2005). Other investigations in Southeast Queensland recorded a prevalence of 14-40% in cracticines in that locality but none of these were described or identified beyond genus level (Adlard et al. 2004). *Haemoproteus spp.* were responsible for the most infections in the *Cracticus* species sampled in this study. Molecular descriptions based on sections of the cyt-*b* gene have identified a further *Leucocytozoon* lineage (GenBank JN792176; Dodge M. et al. 2011), two *Haemoproteus* lineages (CRAQU01; Beadell et al. 2004; GenBank JN792186; Dodge M. et al. 2011) and a *Plasmodium* lineage (CRAQU02; Ewen et al. 2012). All were found in Black Butcherbird (*C. quoyi*) hosts that were sampled in Papua New Guinea and northern Australia. Parasite morphological features were not identified as a part of these molecular studies. To our knowledge, this is the extent of published material on the haemosporida in *Cracticus* species.

The fragmentary knowledge of haemosporidian parasites of *Cracticus* species is exacerbated by the two methodologies used for their identification. For instance, it is not known whether the *Leucocytozoon sp.* found in Cracticus hosts in southern Queensland by Peirce et al. (2005) is the same as that found in *C. quoyi* (PNG) by Dodge M. et al. (2011) because complementary molecular and morphological descriptions of the parasites are lacking. Here we present a morphological and molecular assessment of a novel *Haemoproteus* parasite found in *C. louisiadensis*, a species endemic to small forested islands in the Louisiade Archipelago of Southeast Papua New Guinea (Fig.1) and considered to be Data Deficient by

the International Union for Conservation of Nature (IUCN 2012). This is the most geographically restricted of the *Cracticus* species. To gain perspective of the avian host-specificity of this new *Haemoproteus* parasite, we also screened a range of sympatric bird species from these islands and compared our findings to parasite data from other more widespread *Cracticus* congeners we captured in Brisbane, Australia. This paper forms a platform for future elucidation of *Haemoproteus spp.* infections in this relatively unstudied yet prominent Australo-Papuan bird group.

2. Methods.

2.1. Sample collection

A range of bird species, including *C. louisiadensis*, were captured using mist-nets during the breeding periods of October –January 2013-2015 in the Louisiade Archipelago of Papua New Guinea (11° 27’ S, 153° 25’ E). *Cracticus spp.* hosts were also targeted for capture during 2015 in Brisbane, Australia (27° 30’ S, 152° 59’ E). Blood samples of approximately 50 µl were collected by brachial venipuncture (27g needle) of the vein crossing the ulna joint on the underside of the wing. Blood was collected via capillary action into 75 µl micro-capillary tubes and ejected into 1.5ml microfuge tubes with 300 µl of lysis buffer for storage (10 mM EDTA pH 8.0, 10 mM TRIS pH 8.0, 20 mM NaCl, 1% SDS). Three blood smears per individual were also taken and air-dried as quickly as possible to retain natural parasite morphology (Valkiūnas 2005). Blood smears were fixed for three minutes in the field in AR grade 100% methanol and, following return from the field, postfixed again in methanol for a minute before staining for 45 minutes with 10% Giemsa’s stain (Gurr improved R66 solution, VWR International, Muruarrie Queensland, Australia).

2.2. Morphological assessment

Visual assessments of blood smears were made following Valkiūnas (2006) and morphological measurements taken following Valkiūnas (2005) to identify morphology, assess prevalence in the host and reduce the potential for false negative or positive assessments from molecular techniques. Blood smears from infected individuals were closely scrutinised for disparities in parasite morphology that might indicate mixed infections. Parasite morphology was assessed using a Leica DM1000 stereomicroscope with Leica MC170 HD camera, and Leica Application Suite V4 with the additional Interactive Measurements Module software.

2.3. Molecular assessment

We extracted DNA from blood samples using a salting out extraction method (Ammonium acetate / ethanol precipitation), modified from Richardson et al. (2001). Briefly, approximately 50 µl of blood/lysis buffer mix was added to 250 µl of extraction buffer (100 mM EDTA pH 8.0, 100 Mm TRIS pH 8.0, 10 Mm NaCl, 0.5% SDS) with 10 µl of Proteinase K (10mg/ml) and digested for 3 hours at 56 °C or overnight at 37 °C. Following addition of 180 µl of 7.5M ammonium acetate samples were chilled at -20 °C for 20 minutes, then centrifuged for 12 minutes at 13,000 RPM. DNA was precipitated via two rounds of cold ethanol, first with 100% followed by a 70% dilution. These were centrifuged at 13,000 RPM for 20 minutes before removing supernantant at each step. Samples were then re-suspended with 150 µl of low TE buffer (10 mM TRIS, 1 mM ETDA, pH 8.0) and left to resuspend overnight at 4°C.

We used a number of PCR protocols as part of this investigation which are detailed in Appendix 1a. We determined the sex of birds using PCR primers 2550F-5'-GTTACTGATTCGTCTACGAGA-3' and 2718R-5'-ATTGAAATGATCCAGTGCTTG-3' developed by Fridolfsson and Ellegren (1999). These PCRs also served to eliminate the possibility that false parasite negatives were due to failed DNA extractions.

For samples that returned positive amplifications from the avian sexing PCR, we carried out a detection PCR to screen for the presence of haemosporidian parasite DNA (*Plasmodium* and *Haemoproteus* spp.) by amplifying a 150bp fragment of the Cytochrome oxidase I gene (COI) using primers MalMito_F1-5'-AGCCAAAAGAATAGAAACAGATGCCAGGCCAA-3' and MalMito_R1- 5'-AGCGATRCGTGAGCTGGGTTAAGAACGTCTTGAG-3' (developed by Anders Goncalves da Silva at Monash University, Victoria, Australia).

For samples that returned positive amplifications from the parasite detection PCR, we amplified a 1000bp fragment of the parasite cyt-*b* gene using primers Prim3_F2-5'ACTGGTGTATTATTAGCAACTTGTTATACT-3' and Prim3_R1-5' GCTTGGGAGCTGTAATCATAATGT (developed by Anders Goncalves da Silva at Monash University, Victoria, Australia).

Where positive infections did not amplify using the above technique, we employed a nested PCR technique following Waldenström et al. (2004) to amplify 485bp of the cyt-*b* gene. Reaction conditions and cycling profiles for the nested PCR followed methods outlined in Clark et al. (2015b). All PCRs were carried out on a GenePro Thermal Cycler (Bioer Technology Co. Ltd., Tokyo, Japan) using Queensland Museum facilities (Brisbane, Australia).

Gel electrophoresis was used to identify successful PCR amplifications from all PCRs. PCR products were sequenced using the primers Prim3_F2 and Prim3_R1 (1000bp fragment) at the Australian Genome Research Facility Ltd (AGRF; Brisbane) or for the smaller 485bp fragment using the reverse primer HAEMR2 (Waldenström et al. (2004) at Macrogen Inc (Seoul, South Korea). Sequences were aligned and edited using the software program MEGA 6.0 (Tamura et al. 2013). These were then compared with sequences lodged with the databases MalAvi (Bensch 2015) and GenBank (Benson et al. 2015).

The phylogenetic relationships of parasite lineages were constructed using Bayesian Markov Chain Monte Carlo (MCMC) analysis in the BEAST software (Drummond et al. 2012). All parasite lineages that have been linked to described morphospecies were included in our phylogenetic analysis to assess whether our lineages formed a monophyletic clade along with *Haemoproteus* lineages previously recovered from *Cracticus* hosts. Single *Haemoproteus* lineages recovered from other south Pacific avian hosts were also included to clarify host-parasite relationships in the region. A General Time Reversible (GTR) substitution model, and a site heterogeneity model of Gamma + Invariant sites with a lognormal relaxed clock (uncorrelated) were used. The process was initiated with a random starting tree and a yule speciation prior. Two runs of 20 million iterations were completed, logging parameters every 1 000 iterations. Ten percent burn-in from each run were removed and combined runs using the program LogCombiner v1.8.2 (Drummond et al. 2012). TreeAnnotator (Drummond et al. 2012) was used to generate a consensus tree and Tracer v1.6.0 (available at http://tree.bio.ed.ac.uk/software/tracer/) was used to visually inspect chains and to generate estimated sample sizes (ESS) for each parameter (with values greater than 200 indicating chain convergence). The consensus tree was edited in FigTree (available at http://tree.bio.ed.ac.uk/software/figtree/). Genetic distances between parasite lineages were assessed using the Jukes-Cantor model in MEGA 6.0 (Tamura et al. 2013).

3. Results

We captured and sampled individuals of 32 different bird species across the study islands, which included 27 *C. louisiadensis* (18 males and 9 females). *C. louisiadensis* were the only hosts screened that carried *H. bukaka* sp. nov.. Individuals were captured across the four island populations of this host species distribution (Fig. 1). Overall infection prevalence with *H. bukaka* sp. nov. was high at 74% (n = 20/27) and parasitaemia within infected hosts ranged from < 0.1- 0.6%. However, on two of the four islands (Panawina and Panatinani; Fig.1), only one of seven (14%) *C. louisiadensis* sampled was infected with *H. bukaka* sp. nov., compared to a prevalence of 80% on Sudest (n = 5) and 100% on Sabara (n = 15). All partial cyt-*b* sequences were identical, representing a novel *Haemoproteus* lineage according to MalAvi and GenBank. A single *Plasmodium* lineage (lineage GRW15; GenBank DQ368380), detected in an individual on Panawina Island, was the only other haemosporidian lineage detected in this host species using molecular methods.

Higher taxonomy (following Valkiūnas 2005)

Phylum: Sporozoa (Leuckart 1879)

Class: Coccidea (Leuckart 1879)

Order: Haemosporida (Danilewksy 1885)

Family: Haemoproteidae (Garnham 1966)

Genus: Haemoproteus (Kruse 1890)

Subgenus: Parahaemoproteus (Bennett et al. 1965)

Morphological description of *H. bukaka* sp. nov.

3.1. Macrogametocytes (Fig.2a-e)

These present greater variation in morphology than microgametocytes. Mature macrogametocytes (Fig.2a-c) never fully encircle erythrocyte nucleus and display consistency in halteridial form (Bennett and Peirce 1988). Gametocytes touch the erythrocyte nucleus along the internal margin. The cytoplasm can appear heterogeneous and amorphous near the erythrocyte envelope. Approximately half (48%, n=55) are in contact with the envelope along the entire outer margin and half (52%) are not. Typically those that are not in continuous contact with the envelope, exhibit a deviation near the middle of the gametocyte (Fig.2a-b). Uneven pigmentation can obscure this margin, making contact hard to discern. The gametocyte outline is smooth to wavy, very rarely amoeboid. Gametocytes are typically positioned medially within the erythrocyte. A small-proportion are situated slightly toward a

pole. Mature gametocytes may come close to, but touch neither pole (40%; Fig.2b), both poles (30%; Fig.2c), or through either positioning or development come very close to both but touch only one (30%: Fig.2a). Parasite nuclei in fully-grown gametocytes are consistently sub-terminal and touching the host cell envelope (Fig.2a-c). Only very rarely might they be terminal or touching the host nucleus. There is usually a single large sub-terminal vacuole touching the envelope in a similar position (same side) at the opposite pole of the gametocyte (Fig. 2a-b). However, variation can occur and up to three or more small vacuoles may be present, including terminally (Fig.2c), and rarely (uncharacteristically), with a small vacuole at the gametocyte's nucleated end terminus. Typically, vacuole/s are located at the non-nucleated end of the gametocyte. Pigment granules are usually oval, occasionally round, generally uniform in size and in the transition between dust-like - medium sized (≤ 0.5 – 0.7µm; following Valkiūnas 2005). Granules usually form loose aggregations around the growing vacuole and parasite nucleus in young gametocytes that end up appearing random in the fully-grown gametocyte. Gametocytes cause moderate lateral displacement of the erythrocyte nucleus.

3.2. Microgametocytes (Fig.2h-l)

Microgametocytes differ from macrogametocytes in the typical sexually dimorphic characters and features (Valkiūnas 2005). They also show greater consistency in form than macrogametocytes. Mature gametocytes are halteridial in form and never completely encircle the erythrocyte nucleus (Fig.2i-k). Gametocytes typically have smooth to wavy ends and touch both the erythrocyte nucleus and envelope along each margin. Most are positioned medially (85%, n=33) with a smaller proportion slightly toward one pole (15%). However, approximately two-thirds (61%) of gametocytes bend around the erythrocyte nucleus narrowly missing touching either pole (Fig. 2j-k). Approximately a third (32%) extend to touch one pole. Occasionally a gametocyte may be in contact with both poles (7%; Fig.2i). When stained with Giemsa, pale pink nucleated material appears to form a large central mass extending from the erythrocyte nucleus to reach the envelope (Fig.2h-l). This often takes a long snaking form, running along the margin of the host-cell nucleus before doubling back. Pale lilac/blue cytoplasm is restricted to each pole of the gametocyte and contains the oval to round pigment granules (≤ 0.5 – 0.7µm). Pigment granules are usually in a clump at both ends but one with the majority of granules and the other with just a few (Fig.2i,k); or the granules are clumped at just one end (Fig.2j). Gametocytes cause moderate lateral displacement of the erythrocyte nucleus.

306

307 3.3. Young and growing gametocytes

308 Young gametocytes appear to be variable in their form and placement. Defining features
309 include opposing vacuole and nucleated material (Fig.2f-g). Growing gametocytes often take
310 on a dumbbell appearance (Fig.2d-e), constricted between the nucleated material and vacuole.
311 Granules usually form loose aggregations around the growing vacuole and parasite nucleus in
312 young gametocytes. Depending on gametocyte placement, contact usually begins with the
313 erythrocyte nucleus before growing outward to the envelope (Fig.2d-e, h).

314

315 Table 1 summarises the morphometrics of gametocytes and infected/uninfected erythrocytes.

316

317 4. Molecular description of *H. bukaka* sp. nov.

318 Partial cyt-*b* sequences extracted from infected type-hosts, *C. louisiadensis*, were identical (n
319 = 20). This new lineage CRALOU01 (GenBank KX100323), representing the proposed
320 morphospecies *H. bukaka* sp. nov., sits within a well-defined monophyletic clade that
321 includes other *Haemoproteus* lineages recovered from members of Cracticinae (Fig.3). We
322 found low genetic variation (3-4 bp) amongst the three members of the clade (≤1.6%; mean
323 1.3% Jukes-Cantor distance). A lineage (CRAQUO01/Genbank AY714192) found in the
324 allopatric *C. quoyi* hosts on mainland PNG (unknown location) was the least genetically
325 different from the type lineage in *C. louisiadensis* (0.8%; Beadell et al. 2004). Interestingly,
326 the other lineage that was also found in Black Butcherbirds on mainland PNG (lowlands
327 around Mt Bosavi) differed by 1.6% from both (CRAQUO01/Genbank AY714192) and the
328 newly identified Louisiade lineage CRALOU01. All lineages within the *Haemoproteus*-
329 Cracticinae clade differ by less than 5% genetic distance, the cut-off figure proposed by
330 Hellgren et al. (2007) to represent distinct morphospecies. Within the parasite phylogeny, the
331 clade containing *H. bukaka* sp. nov. was closest to the primary clade representing the
332 Australo-Papuan *H. ptilotis* (4.1 % among-clade mean genetic distance), the clade containing
333 *H. balmorali*/*H. attenuatus* (4.3% among-clade mean genetic distance) and a clade restricted
334 to Australo-Papuan host species from the genus *Myzomela* (5.3% among-clade mean genetic
335 distance). The full, un-collapsed phylogenetic tree can be found in Appendix 1.

336

337 5. Taxonomic summary

Etymology. The species name *bukaka* is proposed as a noun in apposition. This represents Bukaka, the local language name for the avian host on Sabara Island.

Type avian host. Tagula Butcherbird *Cracticus louisiadensis* Tristram 1889 (Passeriformes, Artamidae).

Type locality. Sabara Island, Louisiade Archipelago, Milne Bay Province, Papua New Guinea (11° 08’ S, 153° 06’ E).

Site of infection. Mature erythrocytes; no other information.

DNA sequence. GenBank accession number KX100323.

Type specimens. Hapantotype number G466189; parahapantotype numbers G466190 and G466191; Lodged in the collection of the Queensland Museum, Queensland, Australia.

Distribution and hosts. Found in the insular endemic host *C. louisiadensis* on islands in the Louisiade Archipelago, Papua New Guinea. It is likely to be representative of a *Haemoproteus* clade that also infects other Cracticid hosts in the Australo-Papuan region.

6. Observations from more widespread *Cracticus* species.

Individuals from closely related *C. nigrogularis* (n = 17), *C. torquatus* (n = 7) and *C. tibicen* (n = 9) from Brisbane (SE Queensland) were screened using the same methods. One *C. tibicen* was infected with gametocytes morphologically congruent to the *H. bukaka* sp. nov., but a clean parasite sequence could not be obtained for molecular determination. A further new *Haemoproteus* lineage was found in *C. nigrogularis* (CRANIG01; GenBank KX100322) which closely aligned (9 bp; base-pairs difference) with lineage PTIVIC02 (GenBank JX021542), recovered from a Victoria's Riflebird (*Ptiloris victoriae*) and Yellow-breasted Boatbill (*Machaerirhynchus flaviventer)* from north Queensland (Zamora-Vilchis et al. 2012). This clade sits near those that include *H. pallidus* (mean genetic distance 2.3%). Furthermore, a novel *Plasmodium* lineage (CRATOR01; GenBank KX100325) was found in both *C. torquatus* and *C. tibicen* in Brisbane, differing 9 bp from lineage CRAQUO02 (GenBank JQ905579) that was found in the congener *C. quoyi* in north Queensland (Ewen et al. 2012).

7. Discussion

We have presented both morphological and molecular evidence to support the proposal of *Haemoproteus bukaka* sp. nov. as a distinct species of haemosporidian parasite. This is the first described species of *Haemoproteus* parasite from the strictly Australo-Papuan bird group, Cracticinae. Moreover, we have identified three new genetic lineages of haemosporidian parasites that infect *Cracticus* species (2 *Haemoproteus spp*., GenBank KX100322-323; 1 *Plasmodium spp*., GenBank KX100325), data that will be useful for contributing to our knowledge of haemosporidian diversity in an understudied bioregion.

The combination of features in *H. bukaka* sp. nov.is similar to both *H. nucleophilus* and *H. quiscalus* /*H. monarchus* when using the morphological character key described by Valkiūnas (2005). Despite some features in common, *H. bukaka* sp. nov. macrogametocytes display both unique features and unique structural arrangement. These include opposing positions of a large vacuole/s (>1 μm; following Valkiūnas 2005) and the subterminal nucleus on the host-envelope.

Vacuoles are a defining yet common feature in many haemosporidian parasites (Valkiūnas 2005). However, the vacuoles in *Haemoproteus* species that sit close to *H. bukaka* sp. nov. (e.g. from Meropidae hosts) are characteristically smaller and randomly placed (e.g. *H. manwelli* Bennett 1978; *H. gavrilovi* Valkiūnas 2005). According to our phylogeny, the most closely related parasite to *H. bukaka* sp. nov. is *H. ptilotis*, a parasite that infects Meliphagid honeyeater hosts in Australia and has previously been described both with and without small vacuoles (Valkiūnas 2005; Clark et al. 2015). These two species can be distinguished in blood smears by the lack of a large vacuole opposing the parasite nucleus in *H. ptilotis* macrogametocytes. Microgametocytes of *H. ptilotis* also have a similar number of pigment granules at both poles, compared with all, or most concentrated at one pole in *H. bukaka* sp. nov.. *Haemoproteus spp.* that have similarly large vacuoles to *H. bukaka* sp. nov. are found in other hosts but these are geographically and genetically distant to the Australo-Papuan Cracticinae. As examples, haematozoa infecting members of Anatidae (*H. macrovacuolatus*; Matta et al. 2014) or Cracidae in South America (*H. ortalidum*; Bennett et al. 1982). The closest morphological match to *H. bukaka* sp. nov. that we have observed to date, is the *Haemoproteus* sp. reported here from the single *C. tibicen* individual in Brisbane (Australia). These gametocytes shared similar placement and morphology, including opposing large

vacuole/s and nuclei on the host cell-envelope. The congeneric host and morphological similarities provide compelling evidence that the infection in *C. tibicen* was likely from the same parasite species or a closely related lineage that sits within the *H. bukaka* sp. nov. clade. Nevertheless, molecular sequence data are needed to confirm this hypothesis.

Just a few base pair differences in haemosporidian cyt-*b* sequences can potentially represent different species (Hellgren et al. 2007). Yet evidence also suggests a 5% genetic distance is likely to result in morphological variation (Hellgren et al. 2007). We found low genetic distances between morphologically different but phylogenetically close species to *H. bukaka* sp. nov.; for example, the *H. ptilotis* clade (4.1%) and the *H. balmorali*/*H. attenuatus* clade (4.3%). We also observed 3-4 bp differences within the clade of *H. bukaka* sp. nov. infecting Butcherbirds. Clearly, even minimal genetic variation may be reflected in morphological changes. Moreover, the variation observed from only a few lineages recorded in *Cracticus* hosts could be evidence for cryptic speciation, a facet of haemosporidian taxonomy that is gaining increasing attention of late (Sehgal et al. 2006; Palinauskas et al. 2015).

The strong avian familial associations often demonstrated for *Haemoproteus* species (Beadell et al. 2004; Valkiūnas 2005; Olsson‐Pons et al. 2015) support our observation of a close genetic relationship between *H. bukaka* sp. nov. and the two lineages previously found in Black Butcherbirds in PNG (*C. quoyi*; JN792186, AY714192). The genetic difference between the two *C. quoyi* lineages in PNG (1.6%) is of note and might be evidence for local host-parasite coevolution due to the high level of divergence and population structure observed in *C. quoyi* populations in PNG (Kearns et al. 2011). This cannot be confirmed due to the lack of locational data for one of the *C. quoyi* lineages and deserves further investigation. However, considering the occurrence of apparent specialist *Haemoproteus* lineages (that only seem to infect a single avian host species) in this bioregion (Clark et al. 2015), we expect these *C. quoyi* lineages will reveal morphological characteristics similar to *H. bukaka* sp. nov.. We also expect future investigations will discover further lineages that are part of this clade infecting Butcherbirds and their allies.

The low parasitaemia levels we observed with *H. bukaka* sp. nov. suggest these were chronic infections (Atkinson 2009). However, these parasitaemia levels were significant given samples were collected during the breeding period when *C. louisiadensis* might be immuno-

compromised and a relapse of parasitaemia might be expected (e.g. Applegate and Beaudoin 1970; Valkiūnas et al. 2004). This parasite species was not identified in any of the other 31 sympatric bird species we sampled in the type locality, showing high prevalence in the one host (74%). This supports the Trade-off Hypothesis (e.g. Drovetski et al. 2014), where overcoming the expected costs of host immune responses might limit host-breadth of a parasite, allowing for high prevalence but reduced physiological cost to the avian host (although see Hellgren et al. 2009). Few other studies from the region have encountered such high prevalence with the exception of those observed in Australasian robins (*Petroicidae*) in north Queensland, Australia (76-82%; Zamora-Vilchis et al. 2012; Laurance et al. 2013). In general, a high prevalence from the region could be considered to be in the vicinity of 50%, and these seem to occur predictably in certain bird groups such as Zosteropids, Ptilonorhynchids and Petroicids (Beadell et al. 2004; Ishtiaq et al. 2010; Ewen et al. 2012; Zamora-Vilchis et al. 2012; Laurance et al. 2013; Olsson‐Pons et al. 2015).

The high prevalence we observed might partially reflect the greater opportunities for infection that are thought to occur year-round in the tropics (Valkiūnas 2005). We do not know the vectors for *H. bukaka* sp. nov., but *Culicoides spp.* biting midges (Ceratopogonidae) are thought to act as vectors for *Haemoproteus spp.* of the subgenus *Parahaemoproteus* (e.g. Valkiūnas et al. 2002; Valkiūnas 2005; Atkinson 2009). In the case of *Culicoides spp.* that feed on people in the type location, local residents report spatial and temporal variation across islands. Consequently, similar patterns in variation might also be expected for *Culicoides spp.* that feed on avian hosts in these islands (e.g. Bensch and Åkesson 2003; Bensch et al. 2007).

We lack data to test whether vector abundance or density differed across islands, and thus cannot assess whether this accounted for the patterns in prevalence we observed. The small island/host sample sizes require caution in interpretation. However, samples were collected during the same time period across islands, removing temporal variation as contributor to the near-absence of *H. bukaka* sp. nov. on the two central islands. Climate, close proximity between islands and forest types are similar across islands, as are the general habitats used by the host, *C. louisiadensis* (WG unpublished data). Furthermore, the high prevalences were identified on both the smallest and largest of the islands within the distribution of the host. This reduces support for an influence of island size/habitat complexity on *H. bukaka* sp. nov.

presence. We suspect host population density may be contributing to the pattern observed, as host density is an important contributor to the persistence of haemosporidian parasites through low transmission periods (e.g. Siraj et al. 2015). The lower population densities of *C. louisiadensis* observed on the two central islands (WG unpublished data) may reduce successful transmission and hence prevalence, or initiate local parasite extinction.

Climate change and associated shifts in vector distributions are an important concern for the conservation of endemic avian species, particularly those that are restricted to small islands in a single archipelago. The threat of impacts on *C. louisiadensis* from the arrival of related hosts carrying novel *Haemoproteus* lineages (e.g. CRAQUO01), i.e. those that infect mainland PNG and Australian congeners, are unknown. Significant divergence across Australia and PNG has occurred amongst other members of the host subfamily Cracticinae, particularly with the fluctuating climates and biogeographical barriers of the Pleistocene (Kearns et al. 2010; Kearns et al. 2011; Kearns et al. 2013). However, the lack of investigations in this host group mean we can only speculate whether the high level of host specificity observed in *Haemoproteus* spp. in the region has also resulted in high divergence of genetic lineages.

We have used morphological and molecular methods to propose the erection of *H. bukaka* sp. nov.. This species currently includes a single genetic lineage (CRALOU01/GenBank KX100323). This parasite demonstrates high host specificity and high prevalence but low parasitaemia in a single endemic host. In light of the dearth of haemosporidian investigations in the Australo-Papuan region, we hope this description will form a platform to promote study of host-parasite relationships in this diverse region.

Acknowledgements

Financial support toward for the fieldwork was kindly provided by the Rufford Foundation, Club 300 bird protection fund and the UQ GPEM School Research Grant. We are grateful to the MalariaRCN for training, D. Gibson for advice, and Georgia Kaipu (NRI), Barnabas Willmott (DOE), and the PNG National Museum and Art Gallery for project sponsorship. Furthermore, Kathryn Hall and Jessica Worthington-Wilmer of the Queensland Museum, for allowing use of their laboratory facilities and offering valuable advice. We are also grateful to D. Mitchell (CI), numerous local landholders and communities in the Louisiade Archipelago for land-access, support and advice. Research was conducted under UQ animal ethics permit

GPEM/172/13/APA (WG), ABBBS 2519 (WG), PNG Department of Environment and Conservation approval WTE2.27.1.1.2 (WG), DAFF Import Permits (WG), PNG NRI Research Visa # 10350017045 (WG), Milne Bay Provincial Government permit (WG). Samples in Brisbane were collected under Department of Environment and Heritage Protection Queensland Government Scientific Purposes Permit # WISP10823212 (SMC).

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749 Table 1: Morphological measurements of *H. bukaka* sp. nov.. All measures are the mean ± SE
750 (range). Nuclear Displacement Ratio (NDR) follows (Bennett and Campbell 1972).

Uninfected erythrocyte (n = 85)	**Host cell**	**Host cell nucleus**	**Gametocyte**
Length (µm)	11.81 ± 0.06 (10.19 - 13.43)	5.91 ± 0.05 (4.88 - 7.02)	-
Width (µm)	7.25 ± 0.05 (6.11 - 8.14)	2.58 ± 0.03 (2.16 - 3.38)	-
Area (μm^3)	66.19 ± 0.56 (53.71 - 78.13)	11.91 ± 0.13 (8.40 - 15.75)	-
Proportion of host cell (%)	-	18.07 ± 0.21 (14.14 - 22.45)	-
Microgametocyte (n = 33)			
Length (µm)	12.86 ± 0.13 (11.36 - 14.54)	5.74 ± 0.08 (4.89 - 6.96)	12.19 ± 0.16 (10.51 - 14.36)
Width (µm)	7.30 ± 0.08 (6.39 - 8.04)	2.37 ± 0.04 (2.03 - 2.73)	2.79 ± 0.08 (2.01 - 3.97)
Area (μm^3)	73.94 ± 1.06 (63.87 - 85.86)	10.75 ± 0.18 (8.63 - 12.98)	34.03 ± 0.91 (26.63 - 46.15)
Proportion of host cell (%)	-	14.55 ± 0.28 (10.91 - 18.33)	46.58 ± 1.33 (32.61 - 63.14)
Pigment granules (n)	-	-	6.86 ± 0.22 (4 -10)
NDR	-	0.84 ± 0.02 (0.60 – 1.03)	-
Macrogametocyte (n = 55)			
Length (µm)	12.95 ± 0.11 (11.12 - 14.97)	5.57 ± 0.04 (4.92 - 6.37)	12.64 ± 0.10 (10.46 - 14.50)
Width (µm)	7.11 ± 0.08 (5.70 - 8.27)	2.36 ± 0.04 (1.68 - 3.10)	2.74 ± 0.09 (1.72 - 4.63)
Area (μm^3)	73.83 ± 0.66 (61.93 - 86.11)	10.34 ± 0.15 (8.38 - 14.33)	38.41 ± 0.72 (27.48 - 49.65)
Proportion of host cell (%)	-	14.06 ± 0.24 (11.26 - 19.82)	52.13 ± 0.97 (38.21 - 67.93)
Pigment granules (n)	-	-	11.01 ± 0.25 (6 - 16)
NDR	-	0.75 ± 0.02 (0.43 - 1.00)	-
Vacuole (μm^3)	-	-	3.64 ± 0.20 (1.49 – 7.96)

751

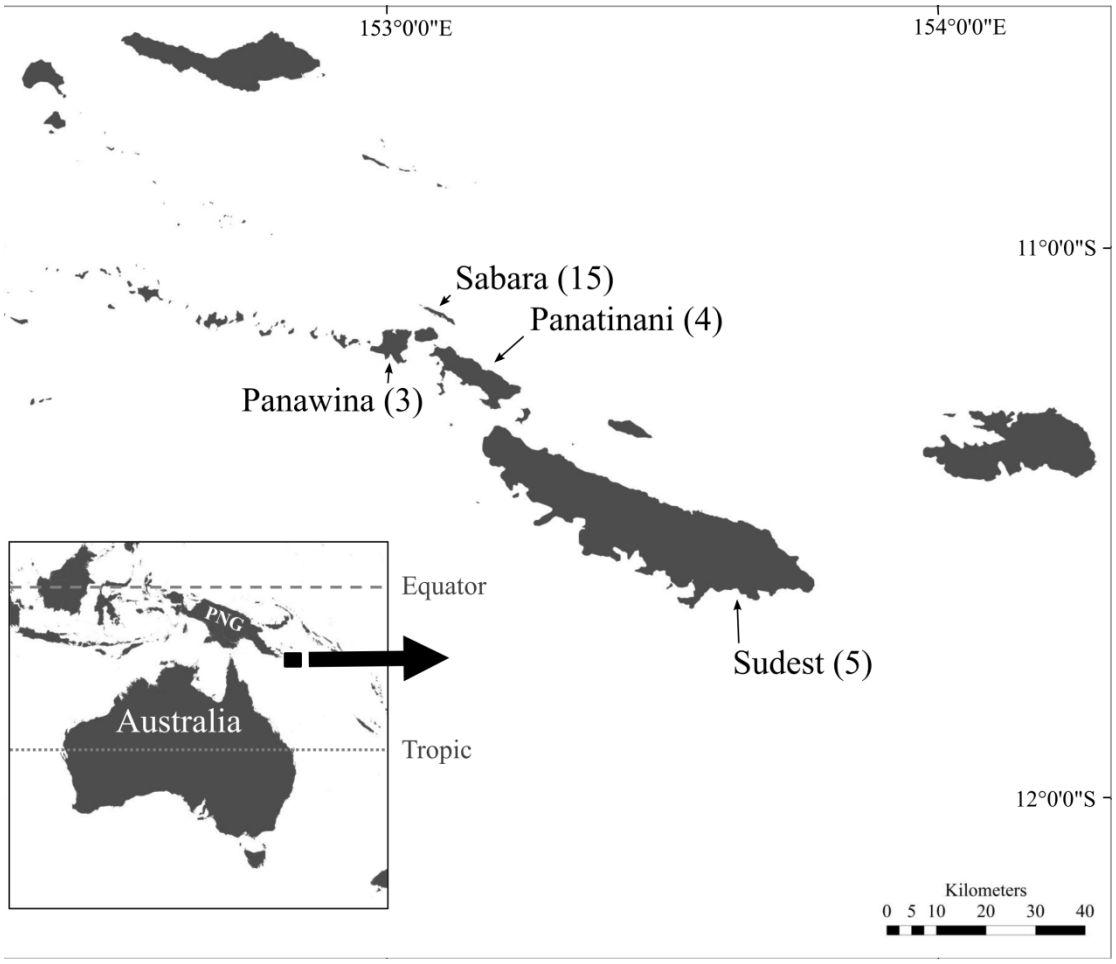


Figure 1. Location of the study islands in the Louisiade Archipelago, Papua New Guinea. The four islands that support the type host *C. louisiadensis* are labelled from the largest, Sudest in the south, to the smallest, Sabara in the north. Sample sizes are in parentheses.

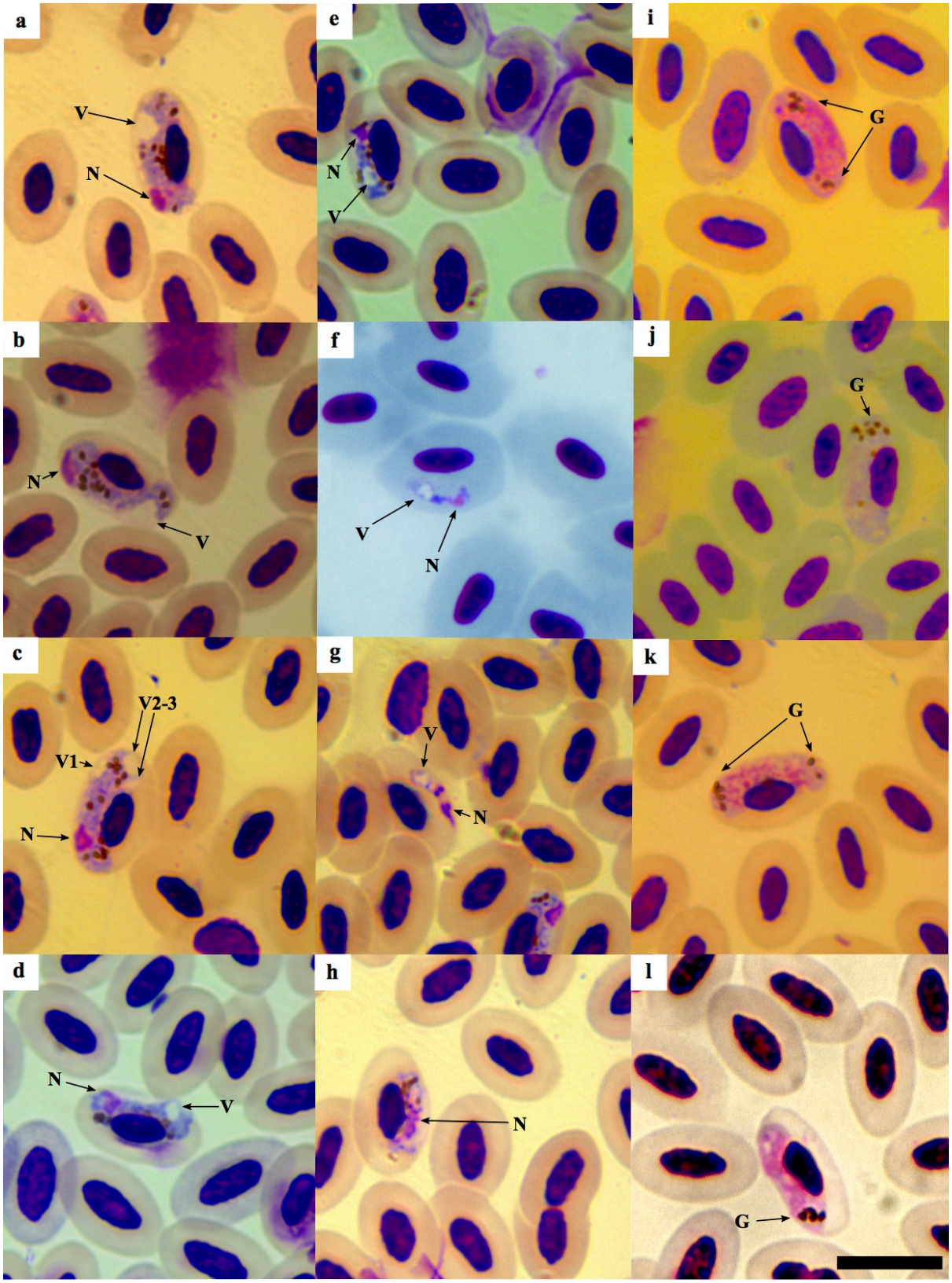


Figure 2. Morphological features of *H. bukaka* sp. nov. gametocytes from multiple individuals of *C. louisiadensis*. N = Nucleus; V = Vacuole; G = Pigment granules. Scale bar shown at (l) bottom right = 10 µm.

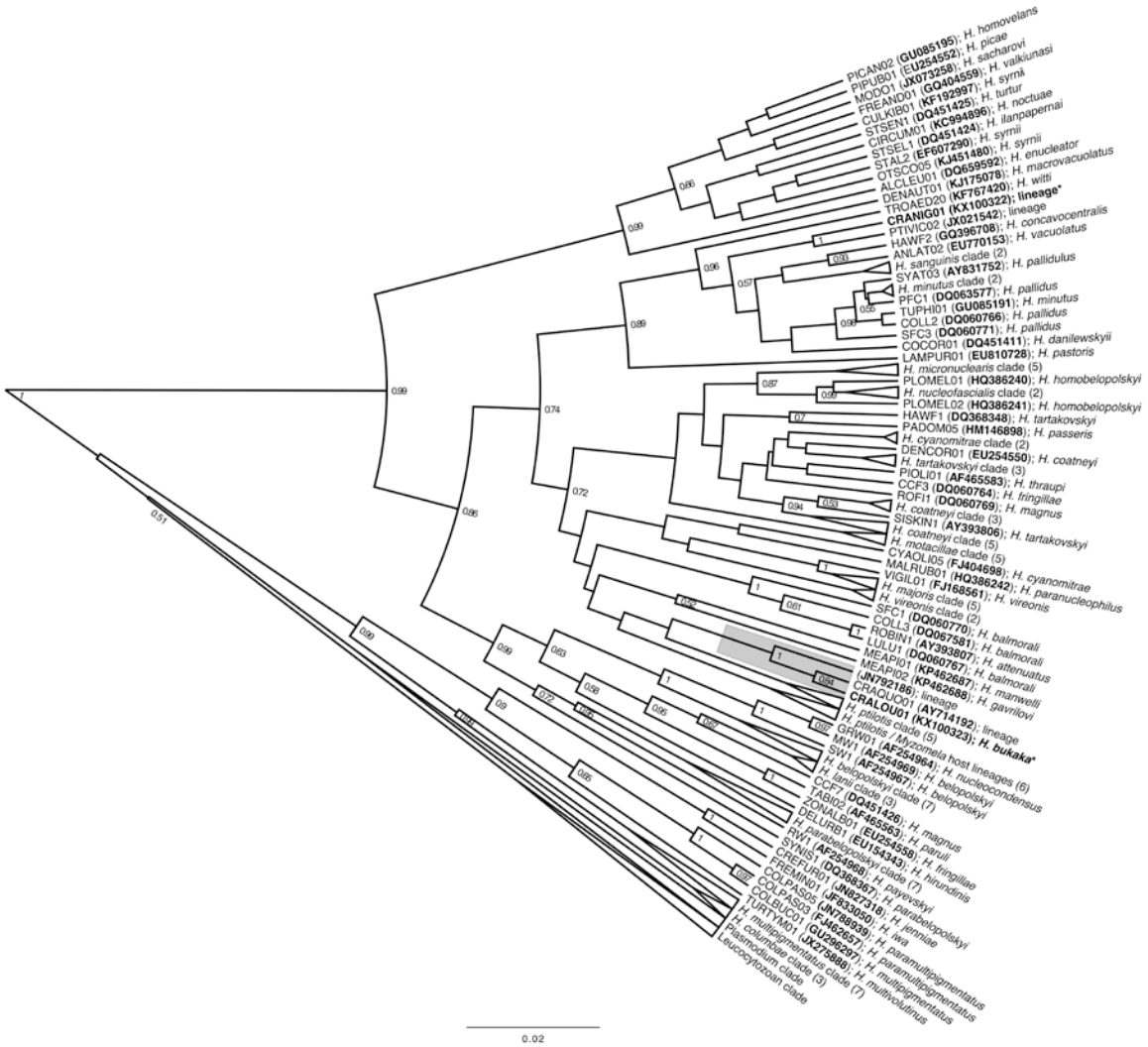
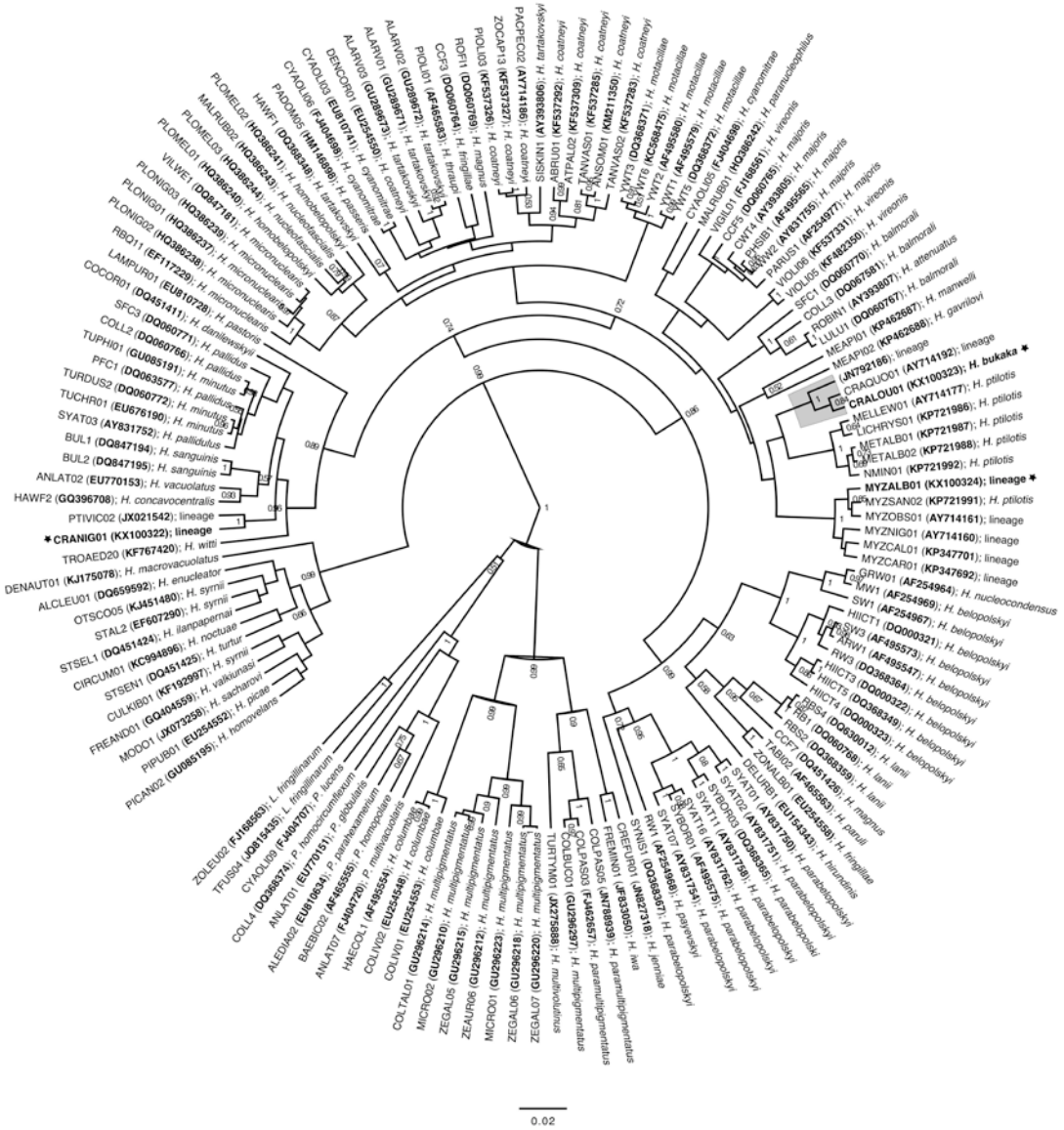


Figure 3 - *0.5 page figure*. Bayesian phylogenetic reconstruction of *Haemoproteus spp.* lineages linked to described morphospecies. Included are MalAvi codes followed by their GenBank accession numbers (in bold in parentheses). Six *Plasmodium* and one *Leucocytozoon* species are included as outgroups. The number of lineages in collapsed clades are given in parentheses. Node labels are Bayesian posterior probabilities ≥ 50%. The node containing the proposed new *Haemoproteus* species is shaded and new lineages identified in the current study are denoted with *. The full figure without collapsed clades is included as Appendix 1b.

773 Appendix 1a. PCR reaction conditions used to screen for avian haemosporidians. TopTaq used = Qiagen TopTaq polymerase mastermix

Purpose	Primer	Sequence	Temperatures	Reaction	Reference
Sexing PCR	2550F	5'-GTTACTGATTCGTCT ACGAGA-3'	94°C (5 min), 35 cycles of 94°C (30 sec), 51°C (40 sec), 72°C (40 sec), 72°C (5 min) final extension	10µl reactions: 5µl TopTaq, 0.75µl each primer (10mM), 2µl H20 and 1.5µl DNA extraction.	Fridolfsson and Ellegren (1999)
Sexing PCR	2718R	5'-ATTGAAATGATCCAGTGC TTG-3'	94°C (5 min), 35 cycles of 94°C (30 sec), 51°C (40 sec), (40 sec), 72°C (5 min) final extension	10µl reactions: 5µl TopTaq, 0.75µl each primer (10mM), 2µl H20 and 1.5µl DNA extraction.	Fridolfsson and Ellegren (1999)
Detection PCR	MalMito _F1	5'-AGCCAAAAGAATAGAAA CAGATGCCAGGCCAA-3'	94°C (5 min): Touchdown; 10 cycles 94°C (30 sec), 66°C (30 sec, drop 1°C per cycle) and 72°C (30 sec); 30 cycles of 94°C (30 sec), 56°C (30 sec), 72°C (30 sec), final extension 72°C (5 min).	13µl reactions: 6.5µl TopTaq, 0.5µl each primer (10mM), 1µl H20 and 4.5µl DNA extraction	Developed by Anders Goncalves da Silva at Monash University, Victoria, Australia
Detection PCR	MalMito _R1	5'-AGCGATRCGTGAGCTGG GTTAAGAACGTCTTGAG-3'	94°C (5 min): Touchdown; 10 cycles 94°C (30 sec), 66°C (30 sec, drop 1°C per cycle) and 72°C (30 sec); 30 cycles of 94°C (30 sec), 56°C (30 sec), 72°C (30 sec), final extension 72°C (5 min).	13µl reactions: 6.5µl TopTaq, 0.5µl each primer (10Mm), 1µl H20 and 4.5µl DNA extraction	Developed by Anders Goncalves da Silva at Monash University, Victoria, Australia
Full cyt-*b* amplification (1000bp)	Prim3_F 2	5'-ACTGGTGTATTATT AGCAACTTGTTATACT-3'	94°C (5 min): Touchdown; 4 cycles 94°C (30 sec), 56°C (45 sec, drop 1°C per cycle) and 72°C (45 sec); 25 cycles of 94°C (30 sec), 53°C (45 sec), 72°C (45 sec), final extension 72°C (5 min).	20µl reactions; 10µl TopTaq, 0.5µl each primer (10mM), 6µl H20, 3µl DNA extraction	Developed by Anders Goncalves da Silva at Monash University, Victoria, Australia
Full cyt-*b* amplification (1000bp)	Prim3_R 1	5'-GCTTGGGAGCTGTAATCA TAATGT-3'	94°C (5 min): Touchdown; 4 cycles 94°C (30 sec), 56°C (45 sec, drop 1°C per cycle) and 72°C (45 sec); 25 cycles of 94°C (30 sec), 53°C (45 sec), 72°C (45 sec), final extension 72°C (5 min).	20µl reactions; 10µl TopTaq, 0.5µl each primer (10mM), 6µl H20, 3µl DNA extraction	Developed by Anders Goncalves da Silva at Monash University, Victoria, Australia
Nested PCR1 (outside reaction)	HAEMN F	5'–CATATATTAAGAGAATTA TGGAG-3'	94°C (3 min): 30 cycles of 94°C (30 sec), 53°C (30 sec), 72°C (45 sec); final extension 72°C (5 min).	20µl reactions; 10µl TopTaq, 0.25µM each primer, 3µl DNA	Waldenström et al. (2004)
Nested PCR1 (outside reaction)	HAEMN R2	5'-AGAGGTGTAGCATATCTA TCTAC-3'	94°C (3 min): 30 cycles of 94°C (30 sec), 53°C (30 sec), 72°C (45 sec); final extension 72°C (5 min).	20µl reactions; 10µl TopTaq, 0.25µM each primer, 3µl DNA	Waldenström et al. (2004)
Nested PCR2 (inside reaction)	HAEMF	5'-ATGGTGCTTTCGATATAT GCATG?-3'	94°C (3 min): 30 cycles of 94°C (30 sec), 51°C (30 sec), 72°C (45 sec); final extension 72°C (5 min).	20µl reactions; 10µl TopTaq, 0.25µM each primer, 0.8µl outside PCR1 product	Waldenström et al. (2004)
Nested PCR2 (inside reaction)	HAEMR 2	5'-GCATTATCTGGATGTGAT AATGGT-3'	94°C (3 min): 30 cycles of 94°C (30 sec), 51°C (30 sec), 72°C (45 sec); final extension 72°C (5 min).	20µl reactions; 10µl TopTaq, 0.25µM each primer, 0.8µl outside PCR1 product	Waldenström et al. (2004)

Appendix 1b.



(*Full page fig*.) The full consensus tree of 142 lineages showing the current 52 *Haemoproteus* species recognised using morphological and molecular methods (Bensch 2015). This Bayesian phylogenetic reconstruction was generated in BEAST. Node values are posterior probabilities ≥ 50%. New lineages are indicated with * and the clade containing the proposed *H. bukaka* sp. nov. is highlighted with shading. Six *Plasmodium* and one *Leucocytozoon* species are included as outgroups.