Molecular and morphological characterization of *Haemoproteus*
(*Parahaemoproteus*) *ptilotis*, a parasite infecting Australian honeyeaters
(Meliphagidae), with remarks on prevalence and potential cryptic speciation

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Abstract

Avian *Haemoproteus* (Haemosporida) parasites occur in birds on all continents apart from Antarctica. Molecular screening techniques have uncovered previously unforeseen levels of *Haemoproteus* lineage diversity; however, fewer than 20% of genetic parasite lineages have been linked to morphological descriptions. The process of linking morphological descriptions to DNA barcodes for *Haemoproteus* spp. is important for the study of host-parasite interactions and the potential for cryptic speciation. Here, we describe cytochrome-*b* barcodes and morphological diagnostics for the identification of *Haemoproteus* (*Parahaemoproteus*) *ptilotis*, a systematically confusing parasite found in Australian honeyeaters (family Meliphagidae). We characterised infections from the original type host (*Lichenostomus chrysops*) as well as from four co-occurring meliphagid species in southeast Queensland, Australia to investigate intraspecific variation in morphology and lineage identity. We recorded eight lineages that grouped into a well-supported monophyletic group, supporting the linkage of the described lineages to *H. ptilotis*. However, comparisons of diagnostics between the type host and co-occurring meliphagid hosts revealed high genetic diversity and variable morphology that could be indicative of cryptic speciation. This study highlights that morphological descriptions alongside molecular characterisation remain crucial if we are to gain an understanding of the true diversity and host-specificity of protozoan parasites in Australia and elsewhere.

Key words: Australia, cryptic species, DNA barcode, *Haemoproteus*, host specificity,

PCR

Introduction

Avian haemosporidians (Haemosporida) belonging to the genus *Haemoproteus* are vector-borne blood parasites found in birds on all continents apart from Antarctica (Valkiūnas 2005). Taxonomic descriptions, that are based largely on diagnostic morphological characteristics of blood stages, have identified over 130 morphospecies (Valkiūnas 2005). However, molecular studies have uncovered previously unforeseen levels of haemosporidian diversity (Bensch et al. 2009, Ishtiaq et al. 2010) and the generation of DNA barcodes using partial cytochrome-*b* (*cyt-b*) parasite sequences is well suited to haemosporidian research due to established polymerase chain reaction (PCR) protocols (Waldenström et al. 2004, Križanauskienė et al. 2010). Moreover, there is some evidence to suggest that *Haemoproteus* lineages whose *cyt-b* sequences differ by as little as a few base pairs may be reproductively isolated entities (Hellgren et al. 2007), yet the vast majority of newly described *Haemoproteus* DNA lineages are only identified to genus level without morphological description (Palinauskas et al. 2013, Valkiūnas et al. 2014).

Morphological analysis of blood smears can yield important information on *Haemoproteus* spp. diversity as well as the competency of hosts, particularly since abortive infections can still yield amplified PCR products (Levin et al. 2013, Valkiūnas et al. 2013). In spite of the powerful research opportunities that PCR affords (Ishtiaq et al. 2008, Palinauskas et al. 2010, Medeiros et al. 2013, Clark and Clegg 2014), morphological descriptions remain crucial to haemosporidian taxonomy (Valkiūnas et al. 2008b, Zehindjiev et al. 2012, Dimitrov et al. 2014). The process of linking morphological descriptions to DNA barcodes for *Haemoproteus* spp. therefore has merit for the ongoing study of host-parasite interactions (Križanauskienė et al. 2006,

Palinauskas et al. 2007, Valkiūnas et al. 2007).

Honeyeaters (Meliphagidae) belong to one of the most widespread and ecologically diverse avian families in the south Pacific (Schodde and Mason 1999, Joseph et al. 2014). Cleland and Johnston (1909) identified a high prevalence of unknown *Haemoproteus* spp. infecting meliphagids in Australia, and originally classified these parasites into three distinct species based on the examinations of material from different avian host species (*Haemoproteus ptilotis*, type host *Lichenostomus chrysops*; *H. philemon*, type host *Philemon corniculatus*; and *H. meliornis*, type host *Meliornis novaehollandiae*). Re-descriptions from original type material as well as new material (host *Manorina melanocephala*) led Bennett et al. (1994) to consider the titles *H. meliornis* and *H. philemon* as synonymous with *H. ptilotis*. The complex was again revisited by Valkiūnas (2005), with modifications from Bennett et al. (1994) and from Laird and Laird (1960) used to determine that morphological variation among host species and / or geography did not warrant the classification of multiple species. Nevertheless, the degradation of original type material for *H. ptilotis* has been a major hindrance to the identification of diagnostic characteristics (Bennett et al. 1994, Valkiūnas 2005). Morphological and molecular characterisation of *H. ptilotis* from the original type host (*L. chrysops*) as well as from additional avian host species using fresh material are necessary to appropriately describe this widespread and systematically confusing parasite. Here, we combine morphological and molecular techniques to characterise *H. ptilotis* from the original type host *L. chrysops*. In addition, we characterise *H. ptilotis* infections from co-occurring meliphagid species to assess morphological and genetic diversity across avian hosts.

Methods

We collected blood samples and blood smears from meliphagid hosts in southeast Queensland, Australia between July 2012 and December 2013 (Table 1). Birds were caught with mistnets, banded with an Australian Bird and Bat Banding Authority band and released at the site of capture after sampling. Blood samples were taken from the wing vein and stored in lysis buffer (1%SDS, 20mM NaCl, 10mM TRIS pH 8.0, 10mM EDTA pH 8.0) for DNA extraction. Thin smears were air dried, fixed in 100% methanol and stained with Giemsa following the protocols in Valkiūnas *et al.* (2008b). We prepared two blood smears for each bird. Smears were examined for *H. ptilotis* infection using light microscopy following the screening protocols in Valkiūnas *et al.* (2008b). We sampled 29 individuals of the original *H. ptilotis* type host, *L. chrysops*, and identified 15 infected individuals. Additionally, we identified *H. ptilotis* infections in 29 of 41 individuals from four co-occurring Meliphagid host species in the study area (Table 1). Prevalence of *H. ptilotis* in Meliphagid host species ranged from 20% (*Meliphaga lewinii*) to 100% (*Manorina melanocephala*; Table 1).

DNA Extraction, PCR and Sequencing

We extracted DNA from blood samples using ammonium acetate/ethanol precipitation (Richardson *et al.* 2001). We amplified a fragment of the parasite cytochrome-*b* (cyt-*b*) gene using a nested-polymerase chain reaction (PCR) protocol (Waldenström *et al.* 2004) following reaction conditions described in (Clark *et al.* 2014a). Amplifications were sequenced on an Applied Biosystems 3130xl Genetic

Analyser (Foster City, California, USA) at the Griffith University DNA Sequencing Facility (Brisbane, Australia).

Phylogenetic analysis

To test whether newly described *H. ptilotis* lineages were monophyletic, we constructed a molecular phylogeny of microscopically confirmed *Haemoproteus* species. We downloaded *Haemoproteus* lineages that have been microscopically assigned to morphospecies from GenBank and the avian malaria database, MalAvi (Bensch et al. 2009). We aligned and edited sequences in Geneious v5.4 (Drummond et al. 2011). We created the phylogeny using BEAST version 1.6.1 (Drummond and Rambaut 2007) with an uncorrelated lognormal relaxed clock and a Yule speciation prior. We used Akaike's information criterion in jModelTest version 0.1.1 (Posada 2008) to determine the appropriate evolutionary model (GTR+I+G). One sequence of *Leucocytozoon* spp. was used as an outgroup. We carried out two runs of 10 000 000 generations, sampling at every 10 000 generations, and used TRACER version 1.6 (available at <http://tree.bio.ed.ac.uk/software/tracer/>) to assess convergence. We removed 10% burnin from each run and combined the two runs using LogCombiner version 1.4.7 (Drummond and Rambaut 2007). MalAvi lineage names (Bensch et al. 2009) and GenBank accession numbers for all parasite lineages used in the phylogeny are presented in Fig. 1.

Morphological analysis

An Olympus BH2 light microscope (Tokyo, Japan) equipped with Nikon Digital Sight camera and NIS-Elements imaging software (Nikon, Tokyo, Japan) was used to

examine slides and to prepare figures. We examined each blood smear for 15–20 min at low magnification (x400), and then at least 100 fields were studied at high magnification (x1,000). For voucher specimens, we screened the entire blood film for the presence of co-infections with other haemosporidian parasites. Blood samples with single infections of *H. ptilotis* were used to identify diagnostic *cyt-b* parasite sequences for morphospecies characterization (DNA barcoding). In addition to the type host and the four co-occurring Meliphagid host species from which we extracted DNA, we also screened smears and performed measurements for infections identified as *H. ptilotis* in *Philemon corniculatus*, using smears that were registered with the International Reference Centre for Avian Haematozoa (IRCAH; accession numbers G463506, G463508).

Results

We confirmed the presence of single infections both by microscopic examination of blood films and by analysis of chromatograms (absence of double peaks in both forward and reverse directions). All of the recorded parasite lineages from meliphagid hosts clustered in a well-supported monophyletic group and could be readily distinguished from other barcoded *Haemoproteus* species on the phylogeny (Fig. 1), supporting the linkage of the described *cyt-b* lineages to the morphospecies *Haemoproteus (Parahaemoproteus) ptilotis*. We identified multiple genetic lineages of *H. ptilotis* that vary in their relative genetic distance to the type lineage (0.6%, LICFRE03 to 5.0%, MYZSAN02), indicating possible intraspecific variation or cryptic speciation across meliphagid hosts.

Morphological description

Haemoproteus (Parahaemoproteus) ptilotis (Cleland and Johnston, 1909). The full morphological measurements for the type host and additional hosts are presented in Table 2.

DNA sequence: Mitochondrial *cyt-b* sequence LICHRY01 (505bp, GenBank accession number KP721986).

Avian host and distribution: The lineage LICHRY01 has only been recorded in the type host, the yellow-faced honeyeater *Lichenostomus chrysops*, in southeast Queensland (QLD), Australia. The following closely related lineages have been recorded in co-occurring Meliphagid hosts in southeast QLD, Australia: lineage LICFRE03 (genetic distance to type lineage 0.6%) was recorded in a white-throated honeyeater *Melithreptus albogularis* in this study and previously in a bridled honeyeater *Lichenostomus frenatus* in northeast QLD, Australia (Beadell et al. 2004); lineage MELLEW03 (genetic distance 0.6%) was recorded in Lewin's honeyeater *Meliphaga lewinii*; lineage NMIN01 (genetic distance 1.2%) was recorded in the noisy miner *Manorina melanocephala*; lineages MELALB01, MELALB02 and MELALB03 (mean genetic distance 1.4%) were recorded in the white-throated honeyeater, *M. albogularis*; lineage MYZSAN02 (genetic distance 5.0%) was recorded in the scarlet honeyeater *Myzomela sanguinolenta*.

Site of infection: Mature erythrocytes.

Representative blood films: Voucher specimens (accession numbers G465701 –

G456705), *L. chrysops*, December 2012, collected in Karawatha Forest, Brisbane, QLD, Australia (by N. Clark and S. Clegg) were deposited in the IRCAH (Brisbane, Australia).

Macrogametocytes (Fig. 2a, b, c, d, e) grow around the nuclei of erythrocytes.

Growing gametocytes initially adhere to the envelope of erythrocytes (Fig. 2a) and later adhere to the nucleus (Fig. 2b). Mature gametocytes enclose and displace the nucleus laterally but do not encircle it completely (Fig. 2c, d). The central area of the pellicle in growing gametocytes frequently does not adhere to the erythrocyte envelope, which causes a 'dip' and gives a dumbbell-like appearance (Fig. 2d). Fully grown gametocytes fill the erythrocytes up to their poles and are closely appressed to both the nucleus and envelope of the erythrocyte (Fig. 2e). Outline is usually even in fully grown gametocytes, but ameboid outgrowths are sometimes seen in growing gametocytes. The nucleus is compact and variable in form, and is either subterminal or submedian in position; it usually does not adhere to the erythrocyte nucleus.

Pigment granules are roundish or oval, of medium (0.5 to 1.0 μm) size, usually scattered throughout the cytoplasm but sometimes concentrated to the poles (on average 13.0 ± 2.0).

Microgametocytes (Fig. 2f). Fully grown gametocytes can markedly displace the nucleus of erythrocytes laterally (Table 2; Fig. 2f). Fully grown gametocytes that completely encircle the nucleus are not observed in the type host but have been observed in additional hosts (*M. sanguinolenta* and *P. corniculatus*). Valutin granules may be present. Pigment granules are roundish or oval, of small (<0.5 μm) or medium (0.5 to 1.0 μm) size, usually concentrated to the poles (on average $11.6 \pm$

2.7). Other features are as for macrogametocytes with the usual sexual dimorphic characters (see Valkiūnas 2005).

Variation across hosts: Diagnostic characteristics were generally similar across the different host species sampled (Table 2). However, fully grown microgametocytes were slightly larger in additional host species than in the type host, and the number of pigment granules was generally lower in additional host species (Table 2). IRCAH accession numbers for smears from additional hosts are G465706 - G465710.

Discussion

We have described diagnostic morphological characteristics and identified molecular markers for *Haemoproteus (Parahaemoproteus) ptilotis*, a widespread and abundant vector-borne parasite that infects numerous host species from the family Meliphagidae (Valkiūnas 2005). Despite the existence of over 1300 unique avian haemosporidian *cyt-b* lineages, molecular barcodes have only been identified for approximately 20% of described morphospecies (Bensch et al. 2009, Valkiūnas et al. 2014). Importantly, this study provides the first linkage of morphospecies to *cyt-b* molecular markers for an avian haemosporidian parasite in Australia (Bensch et al. 2009), despite the relatively high prevalence and diversity of *Haemoproteus* spp. infections reported in the Australasian region (Adlard et al. 2004, Beadell et al. 2004, Zamora-Vilchis et al. 2012, Clark et al. 2014b). Phylogenetic trees constructed using the parasite *cyt-b* gene often lend support to previous morphological descriptions (Hellgren et al. 2007, Palinauskas et al. 2007), as was the case in this study. However, we observed slight differences in gametocyte morphology across meliphagid hosts

and recorded eight unique *cyt-b* lineages from only four host species, suggesting that cryptic speciation is a possibility for this parasite.

The honeyeater family is widespread and ecologically diverse in the south Pacific (Driskell and Christidis 2004, Joseph et al. 2014), which could facilitate speciation for host-specialist parasites that reach high levels of prevalence (Poulin and Mouillot 2003, Poulin 2014). Recent evidence suggests that species of *Haemoproteus* often display high levels of specificity with respect to their avian hosts (Beadell et al. 2009, Ventim et al. 2012). Our data shows a high level of host-conservatism for *H. ptilotis* lineages infecting the sampled meliphagid host species, with only one host species presenting multiple unique parasite lineages (*M. albogularis*). Given that the sampled hosts all co-occur in southeast Queensland, this could be interpreted as evidence of cryptic speciation within the *H. ptilotis* complex in meliphagid hosts (Bensch et al. 2004, Sehgal et al. 2006). However, if reproductive isolation has occurred recently, we may not expect to find consistent differences in parasite morphology. For instance, in this study we identified subtle differences in the tendency for full enclosure of *H. ptilotis* microgametocytes across host species, a feature that has previously led to some confusion in nominating species for the *H. ptilotis* complex (Cleland and Johnston 1909, Lederer 2000, Valkiūnas 2005). In addition, the number of pigment granules in both macro- and microgametocytes is variable across the small number of host species sampled here, a pattern that has also been recognized previously for *H. ptilotis* (Mackerras and Mackerras 1960, Bennett et al. 1994, Lederer 2000). Even small variations across *cyt-b* sequences have been linked to different parasite morphospecies (Hellgren et al. 2007), and rigorous microscopic examination combined with PCR has already revealed instances

of cryptic speciation (Palinauskas et al. 2014), an important issue to consider when estimating the true distribution and diversity of haemosporidians (Sehgal et al. 2006). Data from additional meliphagid hosts and vector host specificity are necessary to perform experimental infections and determine whether different host species do indeed harbor reproductively isolated parasite lineages.

We found a high prevalence of *H. ptilotis* infections in *L. chrysops* as well as in the additional meliphagid host species, with the exception of *M. lewinii*. While few additional haemosporidian studies have been carried out in Australia (Clark et al. 2014b), two recent studies also found relatively high prevalence in meliphagid hosts, again with the exception of *M. lewinii* (Zamora-Vilchis et al. 2012, Laurance et al. 2013). The sampled meliphagid species in this study are all omnivorous and are capable of utilising a range of forest strata (Schodde and Mason 1999), which may leave them susceptible to acquiring infectious disease by making vector avoidance difficult (Hart 1990, Fecchio et al. 2013). In addition, *L. chrysops* is locally nomadic in southeast QLD (Clarke et al. 2003), a behaviour that may lead to dispersal-driven accumulation of vector-borne pathogens (Pérez-Tris and Bensch 2005, Knowles et al. 2013). Nevertheless, the low prevalence of *Haemoproteus* infections in *M. lewinii* suggests that certain aspects of the host's ecology, immunocompetence or behaviour towards vectors may result in reduced parasite prevalence (Lima et al. 2010, Fecchio et al. 2013). For instance, the restriction of *M. lewinii* to primarily wet forests in eastern Australia (Moran et al. 2004) may prevent high levels of exposure to the specific vectors responsible for *H. ptilotis* transmission. Given the paucity of avian haemosporidian research in Australia (Valkiūnas 2005, Clark et al. 2014b), future studies are necessary to shed light on the complex interactions between *H.*

ptilotis and meliphagid hosts.

In conclusion, we have identified morphological and molecular diagnostic characteristics for *Haemoproteus (Parahaemoproteus) ptilotis* infecting Australian meliphagid honeyeaters. In addition, we have provided preliminary evidence for the possibility of cryptic speciation in this diverse host-parasite system. As molecular studies continue to identify new levels of haemosporidian diversity, traditional parasitology techniques will become even more relevant to identifying cryptic species and describing parasite distributions. The use of morphological descriptions alongside molecular characterisation remains crucial if we are to gain an understanding of the true diversity and host-specificity of protozoan parasites in Australia and elsewhere (O'Donoghue and Adlard 2000, Adlard et al. 2002, Valkiūnas et al. 2008a, Valkiūnas et al. 2014). The final publication is available at Springer via: [http://dx.doi.org/ 10.1007/s00436-015-4380-8](http://dx.doi.org/10.1007/s00436-015-4380-8).

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Table 1: Sample size and prevalence of *Haemoproteus ptilotis* in Meliphagid hosts in southeast Queensland. The type host is listed in boldface. *No parasite DNA was available from the host *P. corniculatus*, smears from this species were included in parasite morphological analysis (IRCAH accession G463506, G463508).

species	site (Lat, Long)	# screened	# infected with <i>H. ptilotis</i>	# unique cyt-b lineages
<i>Lichenostomus chrysops</i>	Brisbane, QLD (-27.64, 153.07)	29	15	1
<i>Manorina melanocephala</i>	Currumbin, QLD (-28.12, 143.41)	16	16	1
<i>Meliphaga lewinii</i>	Currumbin, QLD	10	2	1
<i>Melithreptus albogularis</i>	Brisbane, QLD	14	10	4
<i>Myzomela sanguinolenta</i>	Currumbin, QLD	1	1	1
<i>Philemon corniculatus</i>	Currumbin, QLD	18	10	NA*

Table 2: Morphological measurements ($\mu\text{M} \pm \text{SE}$) for blood stages of *Haemoproteus ptilotis* infecting the type host (*Lichenostomus chrysops*) and additional meliphagid host species (*Manorina melanocephala*, *Meliphaga lewinii*, *Melithreptus albogularis*, *Myzomela sanguinolenta*, *Philemon corniculatus*). NDR, nucleus displacement ratio (Valkiūnas 2005). N = 25 for all measurements.

Feature	<i>L. chrysops</i>	Additional hosts
Uninfected erythrocyte		
Length	9.1-10.7 (9.9 ± 0.6)	9.8-11.2 (10.4 ± 0.4)
Width	4.6-5.8 (5.4 ± 0.4)	4.5-6.6 (5.8 ± 0.7)
Area	44.9-62.2 (53.6 ± 4.9)	46.3-71.5 (60.1 ± 7.0)
Uninfected erythrocyte nucleus		
Length	4.3-5.6 (5.0 ± 0.4)	4.6-5.9 (5.2 ± 0.4)
Width	1.8-2.8 (2.2 ± 0.3)	2.0-2.6 (2.3 ± 0.2)
Area	9.3-12.8 (11.0 ± 1.1)	10.0-13.8 (11.8 ± 1.2)
Macrogametocyte		
Infected erythrocyte		
Length	10.7-12.2 (11.5 ± 0.6)	11.2-12.4 (11.7 ± 0.5)
Width	4.6-6.0 (5.3 ± 0.4)	4.1-7.2 (5.6 ± 0.9)
Area	52.2-69.7 (61.7 ± 5.1)	48.9-74.5 (64.7 ± 6.8)
Infected erythrocyte nucleus		
Length	4.5-5.9 (5.1 ± 0.6)	4.3-5.7 (4.9 ± 0.4)
Width	1.9-2.4 (2.2 ± 0.2)	1.9-2.5 (2.1 ± 0.2)
Area	8.9-12.7 (11.2 ± 1.0)	9.0-11.6 (10.4 ± 0.9)
Gametocyte		
Length	9.8-11.9 (10.9 ± 0.9)	8.9-11.9 (11.0 ± 1.2)
Width	1.3-2.3 (1.9 ± 0.3)	1.4-3.0 (2.2 ± 0.6)
Area	16.0-26.5 (20.6 ± 3.6)	16.6-34.2 (23.9 ± 5.6)
Pigment granules	10.0-18.0 (13.0 ± 2.0)	6.0-12.0 (9.6 ± 1.6)
NDR	0.6-1.0 (0.8 ± 0.1)	0.6-1.0 (0.8 ± 0.1)

Microgametocyte

Infected erythrocyte

Length	10.9-13.0 (11.8 ± 0.7)	10.6-12.2 (11.5 ± 0.7)
Width	4.8-6.7 (5.6 ± 0.5)	4.6-7.2 (5.7 ± 1.0)
Area	57.3-86.7 (65.8 ± 7.4)	54.2-87.4 (66.3 ± 9.5)

Infected erythrocyte nucleus

Length	4.6-5.9 (5.0 ± 0.5)	4.6-5.4 (5.0 ± 0.3)
Width	1.9-2.6 (2.2 ± 0.2)	2.0-2.6 (2.2 ± 0.2)
Area	9.0-13.8 (11.1 ± 1.3)	9.5-14.1 (10.9 ± 1.3)

Gametocyte

Length	10.3-12.5 (11.7 ± 0.8)	9.7-19.1 (13.9 ± 2.5)
Width	1.3-2.9 (2.1 ± 0.6)	1.7-3.2 (2.3 ± 0.7)
Area	13.3-32.7 (24.3 ± 5.6)	19.4-37.2 (28.8 ± 5.4)
Pigment granules	6.0-16.0 (11.6 ± 2.7)	6.0-10.0 (8.4 ± 1.4)
NDR	0.5-1.0 (0.7 ± 0.1)	0.5-0.8 (0.7 ± 0.1)

Figure legends:

Figure 1: Bayesian molecular phylogeny of *cyt-b* *Haemoproteus* lineages linked to morphologically described species. Presented are MalAvi lineage names, GenBank accession numbers and the linked morphospecies. Shaded area represents lineages linked to *Haemoproteus ptilotis* in Australian Meliphagids. Numbers at nodes represent Bayesian posterior probabilities of branch placement.

Figure 2: *Haemoproteus ptilotis* (cytochrome-*b* lineage LICHRY501) from the blood of *Lichenostomus chrysops*. (a-c), developing gametocytes; (d,e), macrogametocytes; (f), microgametocyte. Arrow, characteristic unfilled space between erythrocyte pellicle and macrogametocyte. Scale bar = 10µm