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Abstract

Haemosporidian parasites of birds are geographically widespread, have been detected in a phylogenetically diverse array of hosts, and have been the focus of extensive research due to both their impacts on birds and their similarity to vector-borne diseases of humans. Advances in molecular diagnostic tools have created a greater awareness of the genetic diversity of haemosporidian infections. Yet in spite of their more or less global distribution, comparatively little is known about the haemosporidians affecting birds in Australia.

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We screened blood from 889 birds (23 species) for haemosporidian blood parasite infections during the 2019 breeding season at Brookfield Conservation Park, South Australia. We examined the genetic (lineage) diversity of haemosporidian infections in this behaviorally and ecologically diverse host assemblage and examined the congruence between parasite and host phylogenies. We identified seven *Haemoproteus* mitochondrial cytochrome *b* lineages, five of which were novel. Four birds had simultaneous co-infections by two *Haemoproteus* lineages each. The *Haemoproteus* lineages clustered at the host family level. Two *Plasmodium* lineages were also identified, each of which had been previously detected in different avian host species in Australasia. We did not detect any Leucocytozoon infections in our sample. This study supplies critical baseline data on host–parasite associations in a poorly-surveyed geographic region.

Keywords Avian blood parasites, *Haemoproteus,* Haemosporida, Mallee, *Plasmodium,* South Australia

Introduction

Avian blood parasites (Apicomplexa: Haemosporida) have attracted substantial research attention since their discovery in the latter part of the nineteenth century. Their global distribution, the taxonomic breadth of the hosts they infect, and their impacts on wild and domestic birds have made them an important focus of study for ecologists, wildlife epidemiologists, and conservationists. Advances in molecular techniques have facilitated a resurgence of field studies of blood parasite infections by permitting rapid, simple, and inexpensive diagnosis of blood parasite infections (Bensch *et al*. 2009). These tools have also fostered a new appreciation for the diversity of blood parasites (Bensch *et al*. 2009; Clark *et al*. 2014).

Both globally and regionally, the diversity of haemosporidian parasites reflects that of their avian hosts (Clark *et al*. 2014; McNew *et al*. 2021). However, broader sampling is needed for unbiased estimation of global patterns of parasite diversity. Extensive work has been done to document the diversity of avian blood parasites, especially in north temperate regions and, more recently, the Neotropics. Relatively few recent broad-scale survey efforts have been conducted in Australia, despite the diversity of its endemic avifauna (Beadell *et al*. 2004; Zamora-Vilchis *et al*. 2012; Laurance *et al*. 2013; Clark *et al*. 2015).

We surveyed avian blood parasites from a community of birds in South Australia. The survey was conducted as part of a larger study relating prevalence of infection in a diverse host assemblage to host behavioral and ecological traits (Hoppe 2021). Here, we report the genetic (lineage) diversity of haemosporidian parasites that we detected in avian hosts and test the congruence of the phylogeny of these lineages with that of their hosts.

Materials and methods

Field methods

We sampled 889 birds of 23 species (Table S1) at Brookfield Conservation Park, South Australia (−34.35°, 139.50°), from August to December 2019. This site is dominated by second-growth eucalypt mallee woodland and open shrubland (Department for Environment and Heritage [DEH] 2005). Over 130 bird species have been observed at Brookfield (Department for Environment and Water [DEW] 2020), with approximately 80 regularly occurring in the park (IRH and AEJ, pers. obs.). Adults (after hatch-year) and juveniles were captured in mist nets using flushing and audio playback. Nestlings of two fairy-wren species (Maluridae: *Malurus splendens* and *M. assimilis*) were sampled at 5–7 days old as part of routine monitoring for those species. Each bird was fitted with a uniquely-numbered metal leg band issued by the Australian Bird and Bat Banding Scheme. We collected whole blood (5–25 µL) from each individual by brachial venipuncture and preserved these samples on WhatmanR FTAR classic cards (GE Healthcare, Chicago, IL) for later analysis. Birds were aged and sexed (when possible) by plumage and soft parts following Hardy (2019). We determined the molecular sex of hatch-year birds and adults of monomorphic species using standard PCR protocols (Griffiths *et al.* 1998; Kahn *et al.* 1998; Lee *et al.* 2010; for details see Supplementary Materials).

Laboratory methods

We extracted DNA from blood spots using DNeasy Blood & Tissue Kits (QIAGEN Inc., Germantown, MD). We used the haemosporidian screening and barcoding protocols described by Eastwood *et al*. (2019) to identify infections. This procedure targets *Plasmodium, Haemoproteus*, and *Leucocytozoon* mtDNA, and amplifies a region of the mitochondrial cytochrome *b* gene used to identify haemosporidian lineages (Bensch *et al*. 2009). We first screened for apicomplexan DNA by PCR with the primers MalMitoF1/MalMitoR1. Samples that tested positive in the initial screening were then barcoded for lineage identification by a nested PCR using external primers Prim3_F2/Prim3_R1 and internal primers HaemNF/HaemNR2 (see Supplementary Materials for details). When the Prim3_F2/Prim3_R1 reaction was successful its product was used as a template for the HaemNF/HaemNR2 reaction. However, samples that failed to amplify with Prim3_F2/Prim3_R1 were still tested with the HaemNF/HaemNR2 primers using genomic DNA as a template. Of the 889 samples tested, 146 were positive for apicomplexan DNA with the MalMitoF1/ MalMitoR1 primers; of those, 77 successfully amplified in the nested PCR and yielded identifiable haemosporidian mtDNA sequences. An additional 10 failed to amplify in the Prim3_F2/Prim3_R1 reaction, but amplified weakly in the HaemNF/HaemNR2 reaction performed on genomic DNA. None of those 10 were successfully sequenced. Samples from 57 birds failed to amplify from either the Prim3_F2/Prim3_R1 or the HaemNF/HaemNR2 reactions, and those from 2 birds with weak amplification signals in the Prim3_F2/ Prim3_R1 reaction failed to amplify in the HaemNF/HaemNR2 reaction with either the Prim3_F2/Prim3_R1 product or genomic DNA. Products from the HaemNF/ HaemNR2 reaction were submitted to the University of Minnesota Genomics Center for sequencing.

We identified lineages by performing BLAST searches of the MalAvi and GenBank databases based on the consensus sequences for each sample (accessed on 15 July 2021). Sequences for novel lineages were submitted to both databases (GenBank accession numbers: MW888415–MW888421; MalAvi lineage names given in **Table 1**). We estimated phylogenetic relationships among haemosporidian lineages using a maximum likelihood approach and assessed the congruence of

Table 1. Haemosporidian lineages identified in birds sampled at Brookfield Conservation Park, Australia in 2019. CYNOV1 and MYZCAL02 belong to the genus *Plasmodium* and all others to *Haemoproteus*. Sample sizes represent adults. Counts of sampled hatch-year birds are given in parentheses. All infected birds were adults. Lineage counts include four cases of co-infection: three ACARUF01/CLIPIC01 and one GAVVIR01/GAVVIR02. One *Malurus assimilis* and one *Ptilotula ornata* had *Haemoproteus* infections that could not be identified to lineage due to poor sequence quality. * Indicates novel lineages.

			Plasmodium		*Haemoproteus*						
Species	N	Infected	CYNOV1	MYZCAL02	POMSUP01*	SMIBRE01*	CLIPIC02	GAVVIR01*	GAVVIR02*	CLIPIC01	ACARUF01*
Malurus assimilis	84 (35)	12	.	.	.	.	11	.	.	.	.
Malurus splendens	83 (52)	15	1	.	.	.	14	.	.	.	.
Acanthagenys rufogularis	14	13	.	.	.	.	.	.	.	9	7
Gavicalis virescens	9	8	.	.	.	.	.	3	3	3	.
Ptilotula ornata	38	23	.	1	.	.	.	.	.	21	.
Melithreptus brevirostris	30	3	.	2	.	.	.	.	.	1	.
Smicrornis brevirostris	100 (1)	1	.	.	.	1	.	.	.	.	.
Pomatostomus superciliosus	39	2	.	.	2	.	.	.	.	.	.

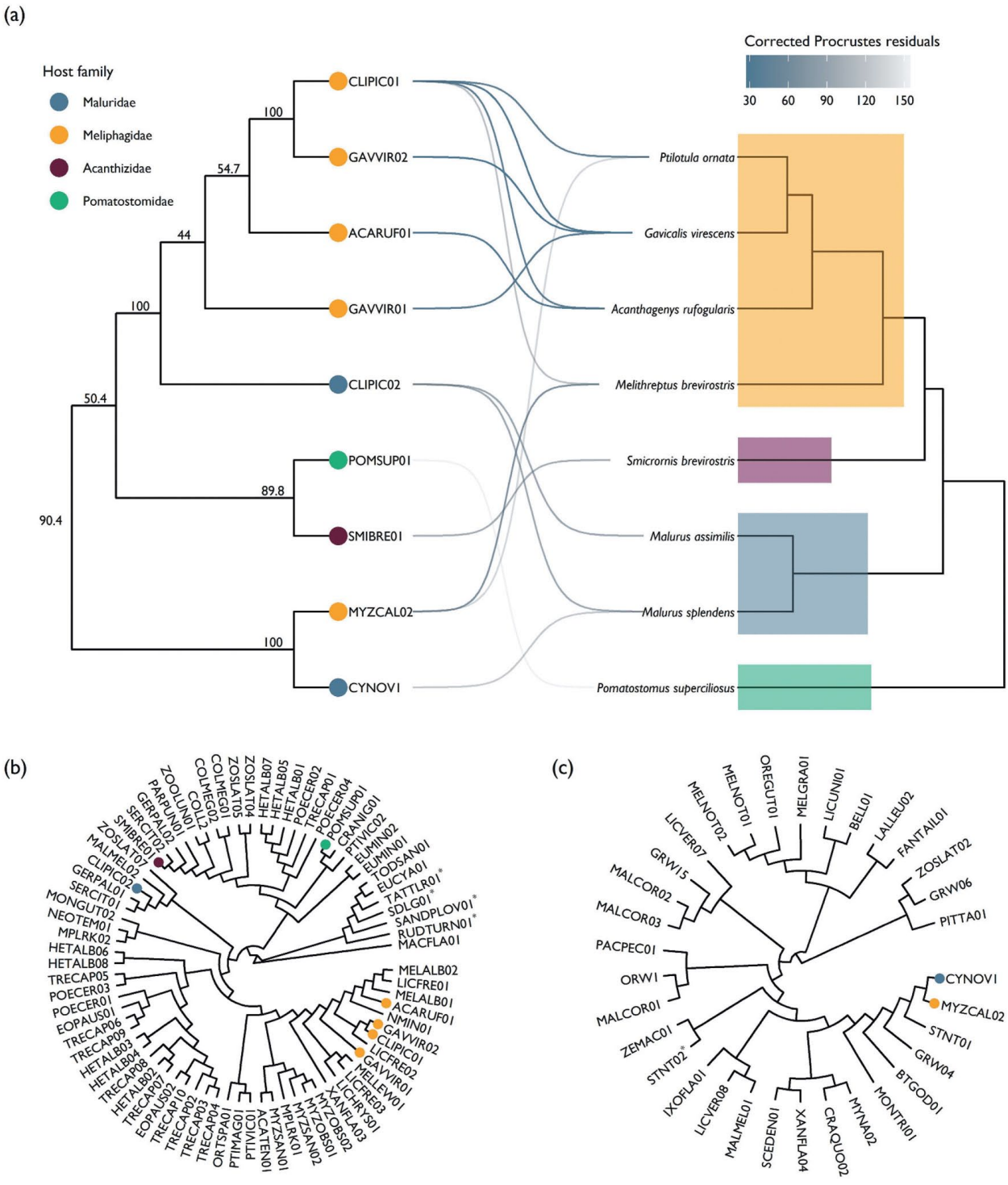
the phylogeny of parasite lineages to that of their hosts using a Procrustean approach to cophylogeny (Balbuena *et al.* 2013; Hutchinson *et al.* 2017). This analysis was performed both for host–parasite associations identified at Brookfield and separately for all *Plasmodium* and *Haemoproteus* cytochrome *b* lineages identified in Australia. All phylogenetic analyses, including alignment and editing of sequencing reads, tree construction, and cophylogenetic analyses were conducted in R (R Core Team 2020; for details see Supplementary Materials).

Results

We found 77 cases of haemosporidian infection in 8 bird species (Table 1, Table S1). All infected birds were adults. We identified 2 *Plasmodium* and 7 *Haemoproteus* lineages (Table 1, Figure 1(a)), with infections being detected throughout the sampling period (Figure 2). No cases of *Leucocytozoon* infection were detected.

We could not confidently characterize *Haemoproteus* infections from one Purple-backed Fairywren (*Malurus assimilis*) and one Yellow-plumed Honeyeater (*Ptilotula ornata*), as the sequences were of poor quality and had several gaps. The fairywren infection was most similar to the *Haemoproteus* lineage found infecting other fairywrens (CLIPIC02; pairwise genetic distance = 6.0%), whereas the honeyeater infection was similar to other lineages infecting honeyeaters (ACARUF01, CLIPIC01; both pairwise genetic distances < 2.0%).

Figure 1. (a) Interactions (observed infections) linking parasite (left) and host (right) phylogenies. Line color indicates the contribution of individual links to the overall congruence between the two phylogenies: links with bolder lines (lower Procrustes residuals) contribute relatively more to the congruence than do those with lighter lines. Maximum likelihood phylogenies for all (b) *Haemoproteus* and (c) *Plasmodium* lineages identified in Australia. Each lineage was isolated from a single host family in the current study (colored points). Trees are based on a 479-bp region of the mitochondrial cytochrome *b* gene and were estimated using generalized time-reversible nucleotide substitution models with gamma-distributed rate variation among sites (a, b, c) and invariant sites (b, c). Annotations in (a) indicate branch support from 1,000 bootstrap replicates. Branch support is not shown in (b) or (c) for visual clarity, but versions indicating estimated branch lengths and support are available in Figures S2 and S3. Lineages marked with * in (b) and (c) are not indexed in MalAvi, but were identified in Australia by Clark *et al.* (2016) and sequences were accessed through GenBank.


(a)
Host family
Maluridae
Meliphagidae
Acanthizidae
Pomatostomidae
Corrected Procrustes residuals
30 60 90 120 150
CLIPIC01
GAVVIR02
ACARUF01
GAVVIR01
CLIPIC02
POMSUP01
SMIBRE01
MYZCAL02
CYNOV1
100
54.7
44
100
50.4
89.8
90.4
100
Ptilotula ornata
Gavicalis virescens
Acanthagenys rufogularis
Melithreptus brevirostris
Smicrornis brevirostris
Malurus assimilis
Malurus splendens
Pomatostomus superciliosus
(b)
(c)

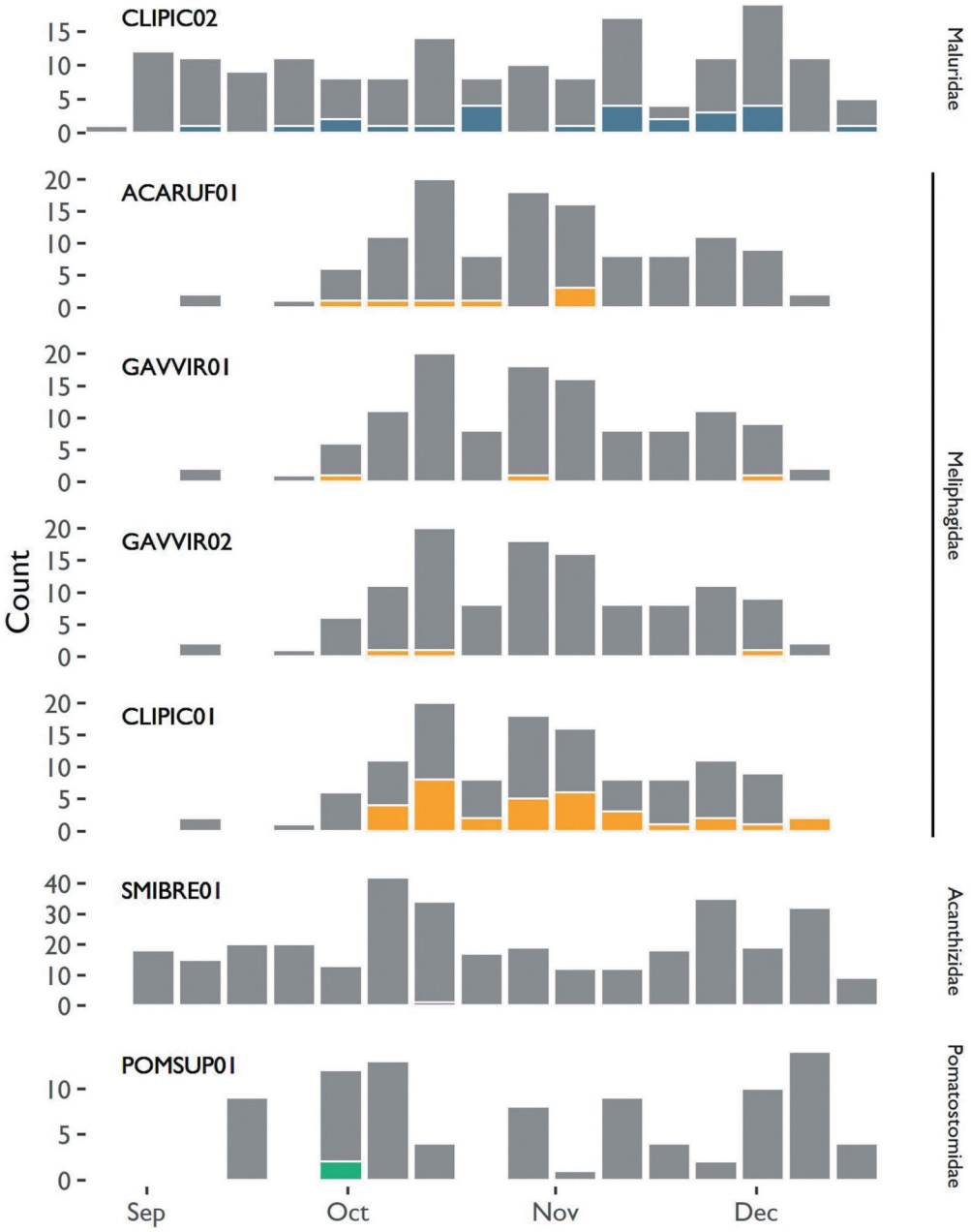


Figure 2. Weekly sampling effort (number of individuals sampled, grey bars) and *Haemoproteus*-positive birds (colored bars; color matches host family). Counts are made separately for each *Haemoproteus* lineage and the host family in which each was identified. Only sampled adults were included. A version of this figure showing the estimated prevalence of each lineage is available in Figure S9.

The cophylogenetic congruence of the parasite lineage phylogeny to that of their associated host species was significantly greater than what would be expected by chance under the null model (see Supplementary Materials for details). This was true both at the local (Brookfield site) level for all parasite lineages combined (sum of squared Procrustes residuals = 25,530.42, P = 0.016; Figure 1(a), Fig. S6) and at the regional (Australia-wide) level for *Haemoproteus* (sum of squared Procrustes residuals = 36,775.1, $P < 0.001$; Fig. S4, Fig. S7) and *Plasmodium* (sum of squared residuals = 40,4207.7, $P < 0.001$; Fig. S5, Fig. S8) parasite–host interactions.

We found *Haemoproteus* lineage co-infections in four birds. Electropherograms from three Spinycheeked Honeyeaters (*Acanthagenys rufogularis*) and one Singing Honeyeater (*Gavicalis virescens*) showed double peaks in the lineage-barcoding region. We resolved the double peaks based on corresponding single infections in other individuals. All three Spinycheeked Honeyeaters were infected with CLIPIC01 and ACARUF01, whereas the Singing Honeyeater was infected with GAVVIR01 and GAVVIR02. We did not detect any *Haemoproteus*/*Plasmodium* co-infections.

Discussion

We report the results of the first survey of haemosporidian parasites of birds at Brookfield Conservation Park, Australia. We identified five novel *Haemoproteus* lineages, two *Haemoproteus* and two *Plasmodium* lineages previously reported from other studies in Australasia, and no cases of *Leucocytozoon* infection (Table 1). The two most commonly-detected *Haemoproteus* lineages (76.9% of infected birds) were identified in previous studies in Australia. However, in both cases the earlier sequences were incomplete in the lineage-barcoding region, and polymorphisms may exist in the unmatched regions. The most common *Haemoproteus* lineage infecting honeyeaters in this community (CLIPIC01) was previously isolated from pardalotes (Pardalotidae: *Pardalotus* spp.) and a Brown Treecreeper (Climacteridae: *Climacteris picumnus*) in Victoria (Balasubramaniam *et al.* 2013). All *Haemoproteus* infections of fairywrens in our system matched a single lineage (CLIPIC02) previously identified in a Brown Treecreeper

in Victoria (Balasubramaniam *et al.* 2013) and two fairywren species (Purple-crowned Fairywren, *M. coronatus*, and Red-backed Fairywren, *M. melanocephalus*) in Western Australia (Eastwood *et al.* 2019). We also found two *Plasmodium* lineages that matched lineages previously identified in New Zealand. Three honeyeaters (Meliphagidae spp.) were infected by *Plasmodium* lineage MYZCAL02, which was previously found in two honeyeater species (Olsson-Pons *et al.* 2015). One Splendid Fairywren (*M. splendens*) was infected by a lineage previously isolated from parakeets (CYNOV1; Ortiz-Catedral *et al.* 2019). While the two *Plasmodium* lineages we identified did separate by host family (Meliphagidae and Maluridae), they are more closely related to one another than either is to any other *Plasmodium* lineage previously found in Australia (Figure 1(c)).

Lineage richness in our sample reflects regional and global patterns. To date, over 1,800 *Haemoproteus* lineages have been identified globally, compared to just over 1,400 *Plasmodium* lineages. In Australia, researchers have isolated 76 *Haemoproteus* lineages and 33 *Plasmodium* lineages (including new lineages identified in this study; Bensch *et al.* 2009; Clark *et al.* 2016). The difference in richness may in part reflect greater host-specificity among *Haemoproteus* spp. (Beadell *et al.* 2004), suggesting a pattern of host–parasite codiversification. Whereas *Haemoproteus* parasites are regularly restricted to taxonomic families or closely-related clades within a host community, *Plasmodium* parasites appear less constrained by host phylogeny, more readily infecting species from different families (Atkinson and Van Riper 1991; Ishtiaq *et al.* 2012; Okanga *et al.* 2014; but see Križanauskienė *et al.* 2006; Svensson-Coelho *et al.* 2013). This pattern was evident in our sample for *Haemoproteus*, with lineages clustering according to the taxonomic family of their hosts (Figure 1(b)), consistent with the idea that these parasites are more host specific.

We found evidence for significant cophylogenetic congruence between the host and parasite phylogenies at both the local and regional levels. The clustering of lineages at the host family level implies that phylogenetically-correlated barriers to transmission are important in shaping cross-community trends in haemosporidian infection. Yet whether these barriers reflect host–parasite, host–vector, or parasite–vector specificity remains unclear (see Martinez-de la Puente *et al.* 2011). Further study of vector distribution and ecology, host

preference, and vector competence for diverse parasite lineages is needed to fully resolve this issue.

Parasite interactions with host immunity can play an important role in the host's susceptibility to secondary infections and can mediate the virulence of multiple infections (Palinauskas *et al*. 2018). Associations between parasites in a host community may indicate facilitation or competition between parasites (Palinauskas *et al*. 2011; Clark *et al*. 2016). Positive associations, which could indicate parasite– parasite facilitation, manifest as a relatively high frequency of co-infections within individual hosts. Four honeyeaters in our sample were co-infected with two *Haemoproteus* lineages each, which could indicate a facilitative interaction involving these lineages. The true prevalence of co-infections may be higher, as PCR-based detection methods underestimate mixed haemosporidian infections (Valkiūnas *et al*. 2006, 2014). However, a larger sample is needed to establish this with certainty.

Our results provide a small but important piece of a global puzzle depicting the distribution and diversity of avian haemosporidian parasites. We contribute critical baseline information for blood parasites from a relatively under-surveyed region, including the identification of five novel parasite lineages and the first records of haemosporidian infection for several host species. This study also highlights the need for further information about vectors, their distribution, and their ecological and coevolutionary interactions with both hosts and haemosporidian parasites.

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Geolocation Data was collected at Brookfield Conservation Park, South Australia (−34.35°, 139.50°).

Data availability All genetic sequences identified in this study are archived in GenBank (ncbi.nlm.nih.gov/genbank/; accession numbers MW888415–MW888421) and MalAvi (130.235.244.92/Malavi/).

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SUPPLEMENTARY MATERIALS

List of avian species sampled

Table S1. Haemosporidian blood parasite infections among species sampled at Brookfield Conservation Park, Australia. Sample sizes represent adults. Counts of sampled hatch-year birds are given in parentheses. All infected birds were adults.

Species	N	*Haemoproteus*	*Plasmodium*	*Leucocytozoon*
Climacteridae				
Climacteris picumnus	2	.	.	.
Maluridae				
Malurus assimilis	84 (35)	12	.	.
Malurus splendens	83 (52)	14	1	.
Malurus leucopterus	0 (1)	.	.	.
Meliphagidae				
Purnella albifrons	26	.	.	.
Acanthagenys rufogularis	14	13	.	.
Gavicalis virescens	9	8	.	.
Ptilotula ornata	38	22	1	.
Nesoptilotis leucotis	3	.	.	.
Melithreptus brevirostris	30	1	2	.
Pardalotidae				
Pardalotus punctatus	7	.	.	.
Pardalotus striatus	6	.	.	.
Acanthizidae				
Acanthiza apicalis	28 (6)	.	.	.
Acanthiza chrysorrhoa	3	.	.	.
Acanthiza uropygialis	105 (25)	.	.	.
Smicrornis brevirostris	100 (1)	1	.	.
Aphelocephala leucopsis	99 (1)	.	.	.
Pomatostomidae				
Pomatostomus superciliosus	39	2	.	.
Pomatostomus ruficeps	51 (1)	.	.	.
Neosittidae				
Daphoenositta chrysoptera	2	.	.	.
Rhipiduridae				
Rhipidura leucophrys	2	.	.	.
Petroicidae				
Petroica goodenovii	31 (4)	.	.	.
Melanodryas cucullata	1	.	.	.
TOTAL	889	73	4	0

Molecular assays

Sexing

For sampled individuals for which sex could not be determined in the field (i.e. nestlings and adults of monomorphic species), we determined the genetic sex by PCR. PCR amplification of the avian chromodomain helicase DNA binding (*CHD*) gene is a fast, accurate, and reliable means of determining an individual's genetic sex, and several assays have been developed to detect different sex-varying portions of the gene. The efficacy of individual assays to determine sex varies between species (Çakmak et al. 2017). We used three different assays, as no test was effective at distinguishing between the sexes for all species (based on individuals of known sex, e.g., females with brood patches). Table S2 provides a listing of the species sexed using each method. A few honeyeaters showed ambiguous results with the CHD1F/CHD1R primers. For those birds, sex was confirmed using the primers 1237L/1272H (Kahn et al. 1998).

All PCR reactions to determine sex were carried out in volumes of 15 µl containing 1× PCR master mix (M7822; GoTaq® G2 Green, Promega Corp., Madison, WI), 0.2 µM of each primer, and 25 ng of DNA template. Reaction conditions are provided in Table S3. Amplification products were separated on a 3% agarose gel under 90 V after 75 (P2/P8 and 1237L/1272H) or 45 (CHD1F/CHD1R) min.

Table S2. Primers used in PCR analyses to determine sex. Each primer pair was used for only a subset of sampled species.

Primer	Primer sequence (5' → 3')	Reference	Species
P2	TCTGCATCGCTAAATCCTTT	Griffiths et al. 1998	Maluridae spp., Acanthizidae spp., Petroicidae spp.
P8	CTCCCAAGGATGAGRAAYTG		
CHD1F	TATCGTCAGTTTCCTTTTCAGGT	Lee et al. 2010	Climacteridae sp., Pardalotidae spp., Pomatostomidae spp., Meliphagidae spp.
CHD1R	CCTTTTATTGATCCATCAAGCCT		
1237L	GAGAAACTGTGCAAAACAG	Kahn et al. 1998	Meliphagidae spp.
1272H	TCCAGAATATCTTCTGCTCC		

Table S3. Cycling conditions for PCR analyses to determine sex.

Primers:	P2/P8	CHD1F/CHD1R	1237L/1272H
Initial heat activation	3 min @ 94°C	4 min @ 94°C	9 min @ 94°C
Number of cycles (*N*):	30	7 + 30[1]	35
Denaturation	60 s @ 94°C	30 s @ 94°C	45 s @ 94°C
N × Annealing	60 s @ 50°C	45 s @ 57–50°C[1]	45 s @ 56°C
Extension	45 s @ 72°C	45 s @ 72°C	45 s @ 72°S
Final extension	5 min @ 72°C	5 min @ 72°C	6 min @ 72°C
Reference	Johnson 2016	Çakmak et al. 2017	Myers et al. 2012

[1]The CHD1F/CHD1R protocol uses a touchdown scheme. The annealing temperature begins at 57°C and drops by 1°C/cycle until reaching 50°C. This temperature is maintained for 30 cycles.

Diagnostic procedure for avian blood parasites

We performed an initial nonspecific screening PCR assay for infection using the primers MalMitoF1 and MalMitoR1 (Table S4). This assay is sensitive to low template concentrations and detects a broad range of haemosporidian parasites, including *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* (see Eastwood et al. 2019), and utilizes less reagent. We performed the screening reactions in volumes of 10 µL containing 1× PCR master mix (M7422; GoTaq® G2 Hot Start Green, Promega Corp., Madison, WI), 0.1 µM of each primer, and 40 ng of DNA template. We included one negative control (nuclease-free water) and one positive control for every 12 samples. As a positive control, we used extracted DNA from a bird previously identified as positive for *Haemoproteus* infection. Cycling conditions are given in Table S5. Products of the screening PCR were resolved on 2% agarose gels under 90 V after 45 min.

Table S4. Primers used in diagnostic PCRs.

Primer	Primer sequence (5' → 3')	Reference
MalMitoF1	AGCCAAAAGAATAGAAACAGATGCCAGGCCAA	Eastwood et al. 2019
MalMitoR1	AGCGATRCGTGAGCTGGGTTAAGAACGTCTTGAG	
Prim3_F2	ACTGGTGTATTATTAGCAACTTGTTATACT	Eastwood et al. 2019
Prim3_R1	GCTTGGGAGCTGTAATCATAATGT	
HaemNF	CATATATTAAGAGAATTATGGAG	Waldenström et al. 2004
HaemNR2	AGAGGTGTAGCATATCTATCTAC	

Samples that were positive under initial, non-specific screening, were then tested to identify parasite lineages present in the sample using the DNA barcoding method described by Eastwood et al. (2019). This protocol is based on the nested PCR approach of Waldenström et al. (2004), but uses the primer pair Prim3_F2 and Prim3_R1 as the external primer pair, which encompasses the primers used by Waldenström et al. (2004), to enhance detection of Australasian haemosporidians. The external primer pair used by Waldenström et al. (2004), HaemNF and HaemNR2, then become the internal primers used to amplify a more specific region that was then used in sequencing. Thus, samples that tested positive in the initial (MalMitoF1/MalMitoR1) screening were further assayed using the nested PCR to amplify a region that includes the ~480 bp barcoding segment of the mitochondrial cytochrome *b* gene of the parasite mtDNA. For the external reaction, we prepared 25 µL reaction volumes containing 1× PCR master mix (M7432; GoTaq® G2 Hot Start Colorless, Promega Corp., Madison, WI), 1.25 µM of each primer (Prim3_F2 and Prim3_R1; see Table S4), and 30 ng template DNA. Reaction conditions are indicated in Table S5. We confirmed amplification by running the products on 1% agarose gels under 90 V for 45 min.

For samples that yielded a clear positive result with the external assay, we used the diluted product as template for the internal reaction (5 µL PCR product in 95 µL nuclease-free water). We still performed PCR of HaemNF/HaemNR2 primers on samples which failed to yield clear positive results with the external assay using diluted genomic DNA (5µL extracted DNA in 45 µL nuclease-free water) to ensure that any samples which failed to amplify with the external primer pair were cross-checked, as both primers should amplify all lineages. The internal reaction mixture was prepared similarly to that for the external reaction, using HaemNF and

HaemNR2 as primers and including 3 µL of the diluted template. Reaction conditions are given in Table S5. Amplified products were resolved on 1% agarose gels under 90 V for 45 min.

Table S5. Cycling conditions for screening and barcoding PCRs.

Primers:	MalMitoF1/MalMitoR1	Prim3_F2/Prim3_R1	HaemNF/HaemNR2
Initial heat activation	15 min @ 95°C	15 min @ 95°C	15 min @ 95°C
Number of cycles (*N*):	5 + 10 + 30[1]	14 + 30[2]	5 + 35[3]
Denaturation	30 s @ 94°C	30 s @ 94°C	30 s @ 94°C
N × Annealing	30 s @ 65–55°C[1]	60 s @ 70–56°C[2]	45 s @ 55–50°C[3]
Extension	30 s @ 72°C	70 s @ 70°C	70 s @ 70°S
Final extension	5 min @ 72°C	5 min @ 70°C	10 min @ 70°C

[1] An annealing temperature of 65°C is used for the first 5 cycles, after which the annealing temperature drops by 1°C/cycle until reaching 55°C (10 cycles). This temperature is maintained for 30 cycles.
[2] Annealing temperature begins at 70°C and drops by 1°C/cycle until reaching 56°C (14 cycles). This temperature is maintained for the remaining 30 cycles.
[3] Annealing temperature begins at 55°C and drops by 1°C/cycle until reaching 50°C (5 cycles). This temperature is maintained for the remaining 35 cycles.

We diluted PCR products from the internal reaction with nuclease-free water and eliminated residual PCR reagents and primers using exonuclease I and shrimp alkaline phosphatase (70995.1.KT; PCR Product Pre-Sequencing Kit, Thermo Fisher Scientific, Waltham, MA, USA). We submitted the cleaned, diluted products to the University of Minnesota Genomics Center (Minneapolis, MN) for bidirectional sequencing using the HaemNF/HaemNR2 primers. Consensus sequences were submitted to the MalAvi and GenBank (accession numbers MW888415–MW888421) databases.

Sequence preparation and construction of parasite phylogeny

Sequencing reads were processed in R using the packages msa, DECIPHER, and sangerseqR (Hill et al. 2014; Bodenhofer et al. 2015; Wright 2016; R Core Team 2020). We aligned forward and reverse reads using the MUSCLE algorithm, a fast and accurate approach to multiple sequence alignment (Edgar 2004a, b). We identified a consensus sequence for each sample from the aligned reads, then aligned these consensus sequences with one another and merged this with

the lineage alignment from the MalAvi database, which we accessed on 15 July, 2021, using malaviR (Ellis et al. 2021). The aligned sample sequences were trimmed to the 479 bp lineage-barcoding region and all base insertions were removed. Sequences with low coverage of the barcoding region were excluded from further analyses (N = 2 individuals of 2 species). We considered those that were identical except for a few ambiguous basecalls (e.g., a thymine residue in one sequence matched to an uncertain pyrmidine in another) to represent the same lineage. To identify lineages, we performed BLAST searches of the MalAvi and GenBank databases using the aligned and trimmed consensus sequences.

We estimated phylogenetic relationships among haemosporidian lineages and constructed trees in R with the packages phangorn and ape (Schliep 2011; Paradis and Schliep 2019; R Core Team 2020). First, we estimated a local phylogeny using all lineages (*Plasmodium* and *Haemoproteus*) identified in this study. We also estimated separate regional phylogenies for all Australian lineages of *Plasmodium* and *Haemoproteus*, respectively, that have sequences in the MalAvi database. A few lineages were identified in Australia by Clark et al. (2016), but are not indexed in MalAvi. These sequences were aligned and merged with the regional datasets prior to phylogenetic analyses. We used a *Leucocytozoon* lineage (PTIVIC03) as an outgroup for all three phylogenies. For each lineage subset, we used AIC_c-based selection to identify the best nucleotide substitution model and compared the resulting maximum likelihood estimates to 1,000 bootstrap replicates to estimate support for branches. A generalised time-reversible (GTR) nucleotide substitution model was selected for all three phylogenies, with gamma-distributed rate variation among sites (i.e. GTR + Γ, all phylogenies) and invariant sites (i.e. GTR + Γ + I, regional phylogenies only). When comparing individual lineages, we computed the pairwise

genetic distances between sequences based on Tamura and Nei's (1993) model of base substitution.

Cophylogenetic analysis

We evaluated the congruence between host and parasite phylogenies both at the local (site) and regional (Australia) level with a Procrustean approach to cophylogeny using the R package paco (Balbuena et al. 2020). This method assesses the global congruence of two linked phylogenies using Procrustean superimposition. It also estimates the contribution of individual links to the overall fit (Hutchinson et al. 2017), thus allowing for comparisons between host and parasite taxa. We tested the significance of the observed phylogenetic concordance against a null model in which host–parasite linkages are shuffled 1,000 times according to the assumptions that parasite evolution tracks host evolution and that the strength of the cophylogenetic signal is driven by the degree of host-specialisation exhibited by parasites ("r1" shuffling algorithm; Hutchinson et al. 2017). The parasite phylogenies were estimated as described above. For the host phylogeny, we sampled 10,000 trees from a published Bayesian phylogeny of bird species (Jetz et al. 2012), pruned these to the set of host species, and identified the maximum clade credibility tree using the package phangorn (Schliep 2011). We computed the pairwise patristic distances for host and parasite trees using package ape (Paradis and Schliep 2019). After performing the superimposition procedure, we evaluated the contribution of each host–parasite link to the overall cophylogenetic fit by examining the bias-corrected Procrustes residuals.

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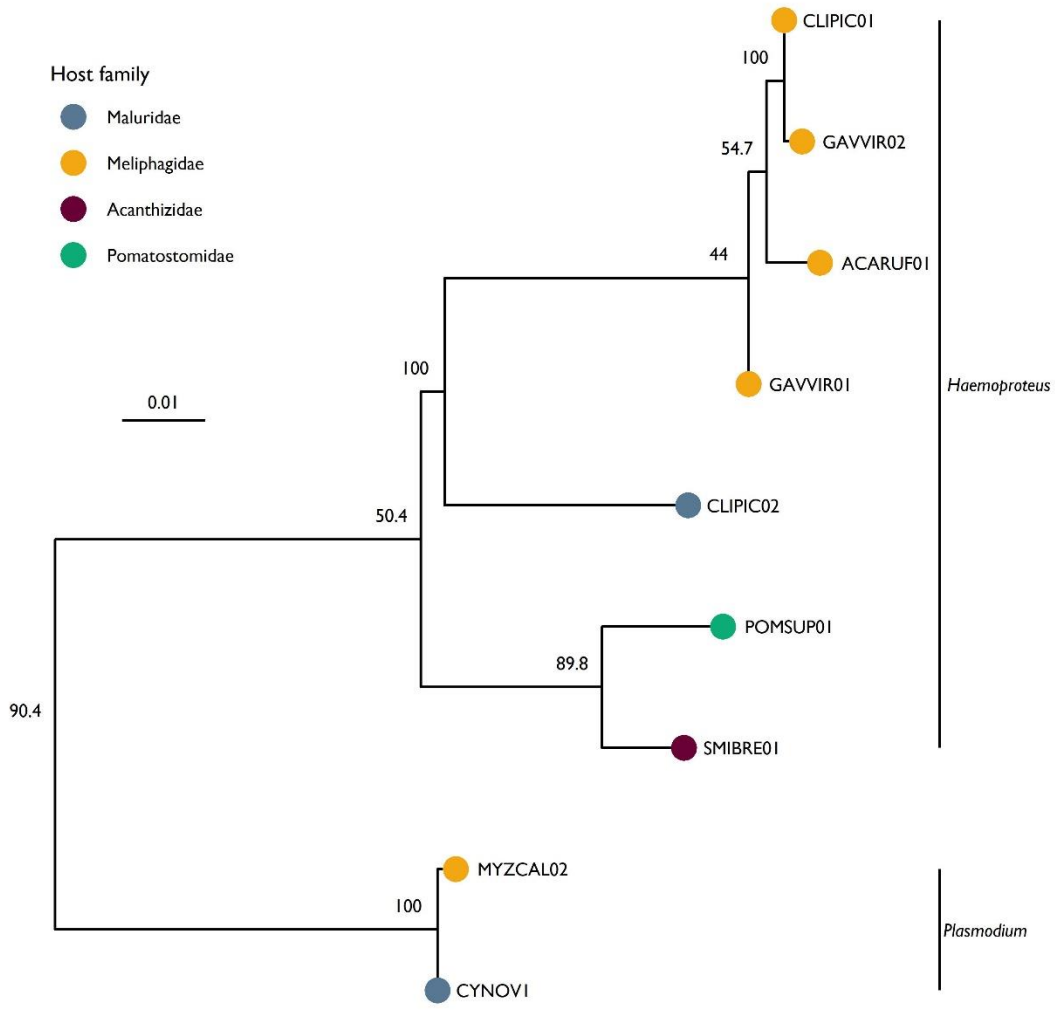

Figure S1, Maximum likelihood phylogeny for haemosporidian lineages identified from avian hosts at Brookfield Conservation Park, South Australia, in 2019. A *Leucocytozoon* lineage (PTIVIC03) was used as an outgroup. Each lineage was isolated from a single host family in the present study (indicated by point colour). The tree is based on a 479-bp region of the mitochondrial cytochrome *b* gene, and was estimated using a generalized time-reversible nucleotide substitution model with gamma-distributed rate variation among sites. Annotations indicate branch support from 1,000 bootstrap replicate trees. The scale bar indicates the branch length corresponding to 0.01 substitutions/site.

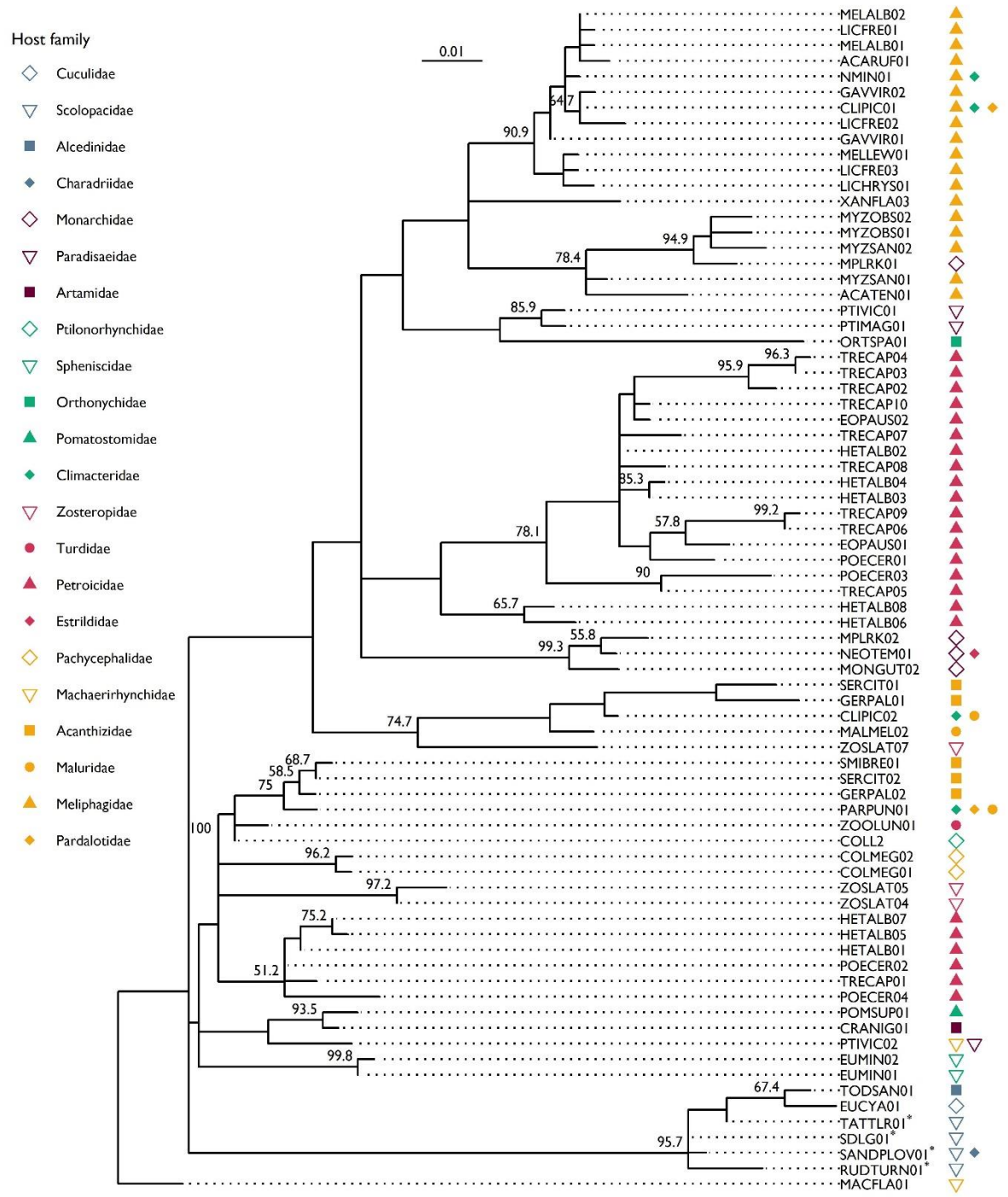

Figure S2. Maximum likelihood phylogeny for all *Haemoproteus* lineages identified in Australia based on a 479-bp region of the mitochondrial cytochrome *b* gene. The phylogeny was estimated using a generalized time-reversible nucleotide substitution model with gamma-distributed rate variation among sites and with invariant sites. Branch length corresponding to 0.01 substitutions/site is indicated by the scale bar. Branch support from 1000 bootstrap replicates is given for branching events receiving support from >50% of replicates. A *Leucocytozoon* lineage (PTIVIC03) was used as an outgroup. Coloured points indicate host families in which each lineage has been reported in MalAvi. Lineages indicated by * were reported by Clark et al. (2016), but are not indexed in MalAvi.

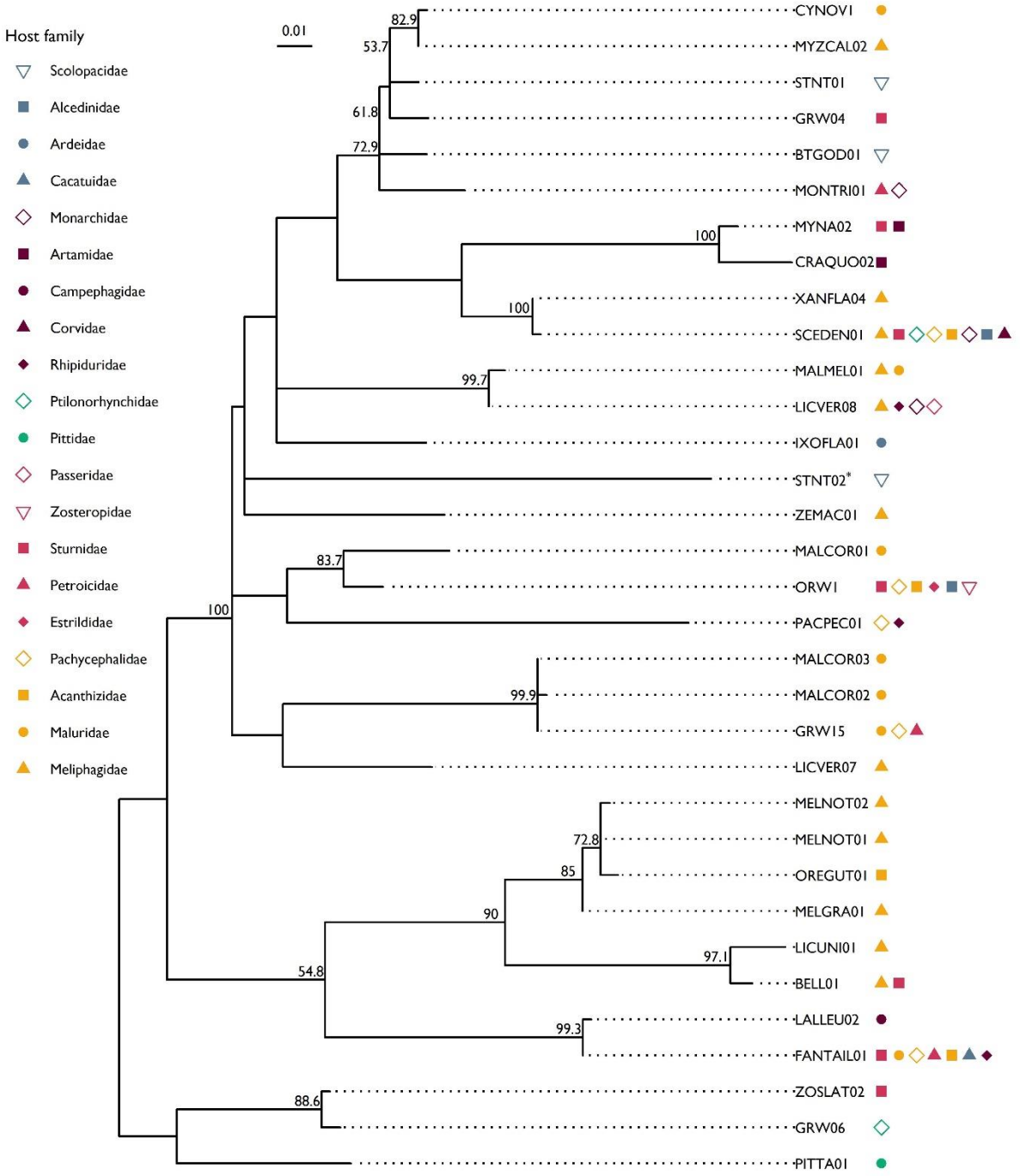

Figure S3. Maximum likelihood phylogeny for all *Plasmodium* lineages identified in Australia based on a 479-bp region of the mitochondrial cytochrome *b* gene. The phylogeny was estimated using a generalized time-reversible nucleotide substitution model with gamma-distributed rate variation among sites and with invariant sites. Branch length corresponding to 0.01 substitutions/site is indicated by the scale bar. Branch support (based on 1,000 bootstrap replicates) is given for branching events receiving support from >50% of replicates. A *Leucocytozoon* lineage (PTIVIC03) was used as an outgroup. Coloured points indicate host families in which each lineage has been reported in MalAvi. Lineages indicated by * were reported by Clark et al. (2016), but are not indexed in MalAvi.

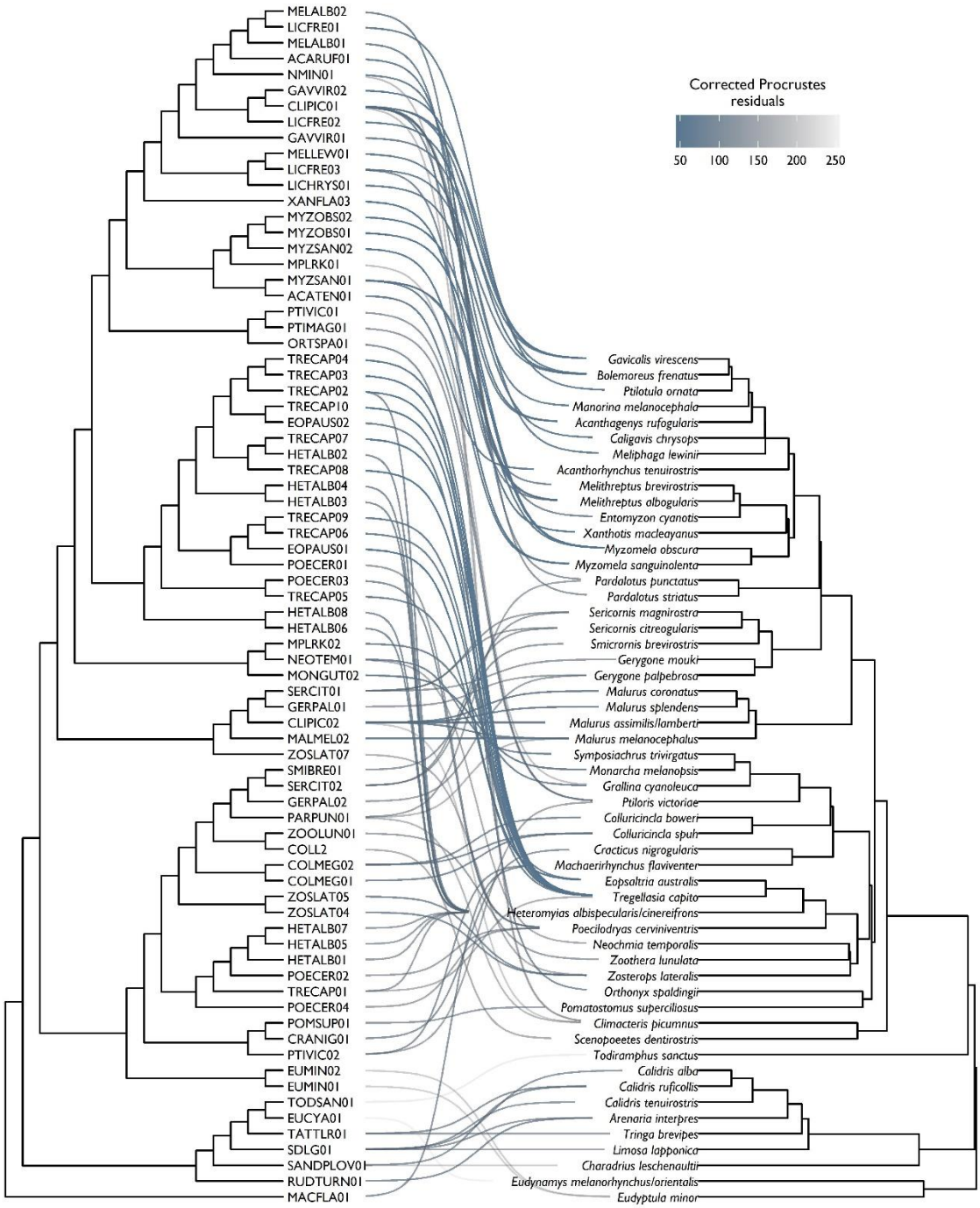

Figure S4. *Haemoproteus* cytochrome *b* lineages (left) and the associated avian host species in which each has been identified in Australia (right). Overall congruence between the host and parasite phylogenies was assessed using a Procrustean approach to cophylogeny (Balbuena et al. 2020). Residuals of the Procrustes superimposition procedure reflect the contribution of individual host–parasite linkages to the overall concordance between host and parasite phylogenies, with links having lower residual values contributing relatively more to the concordance.

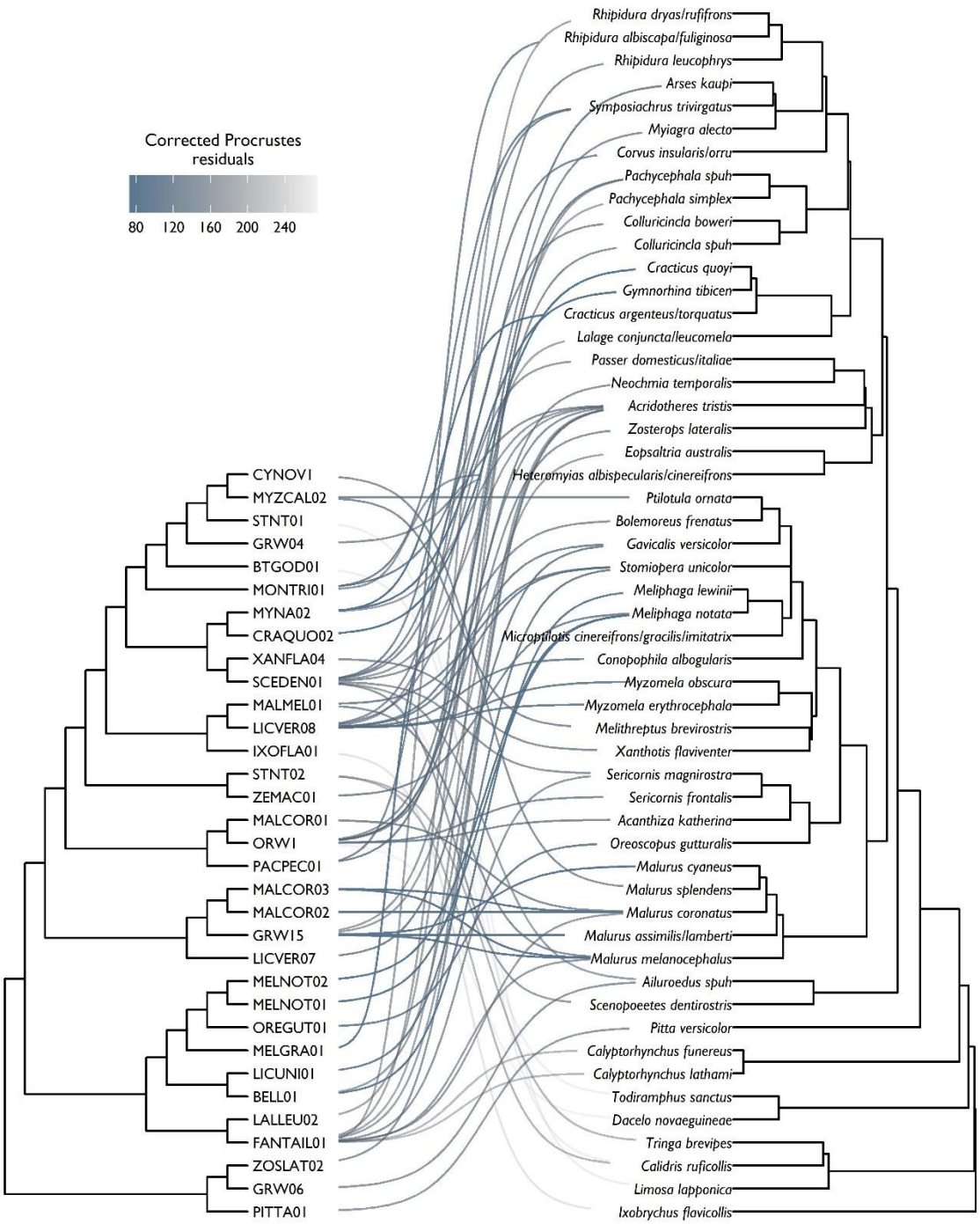

Figure S5. *Plasmodium* cytochrome *b* lineages (left) and the associated avian host species in which each has been identified in Australia (right). Overall congruence between the host and parasite phylogenies was assessed using a Procrustean approach to cophylogeny (Balbuena et al. 2020). Residuals of the Procrustes superimposition procedure reflect the contribution of individual host–parasite linkages to the overall concordance between host and parasite phylogenies, with links having lower residual values contributing relatively more to the concordance.

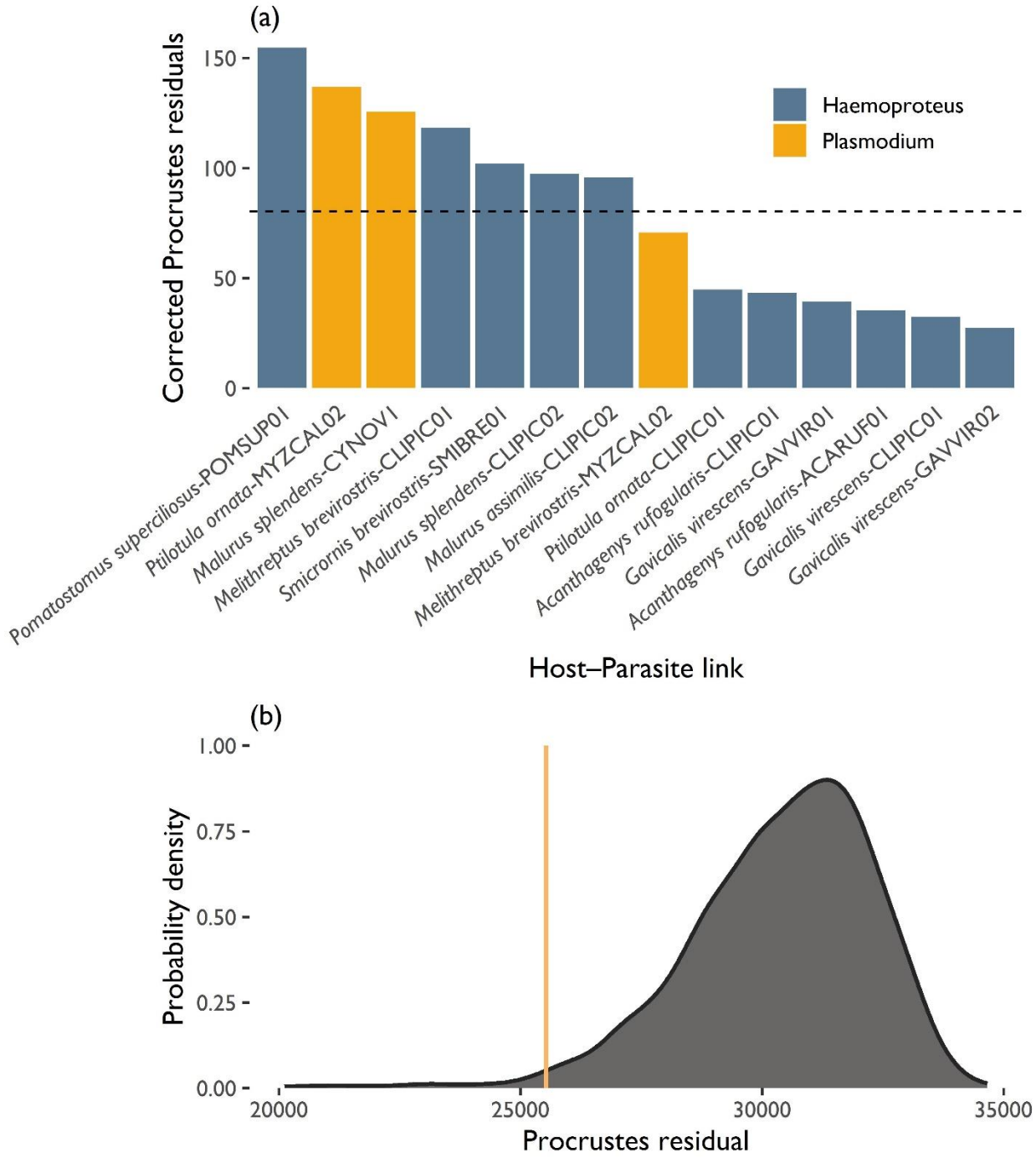

Figure S6. (a) Contributions of individual host–parasite linkages to the overall concordance between host and parasite phylogenies for those associations identified at Brookfield Conservation Park, South Australia, in the present study. Linkages with lower Procrustes residuals contribute relatively more to the concordance. The horizontal dashed line corresponds to the mean residual value across all linkages. **(b)** The overall congruence observed between the host and parasite phylogenies was significantly different from that expected under a null model in which the evolution of parasite lineages tracks that of host species and the cophylogenetic signal is determined by the degree of specialism or generalism of the parasite (P = 0.016).

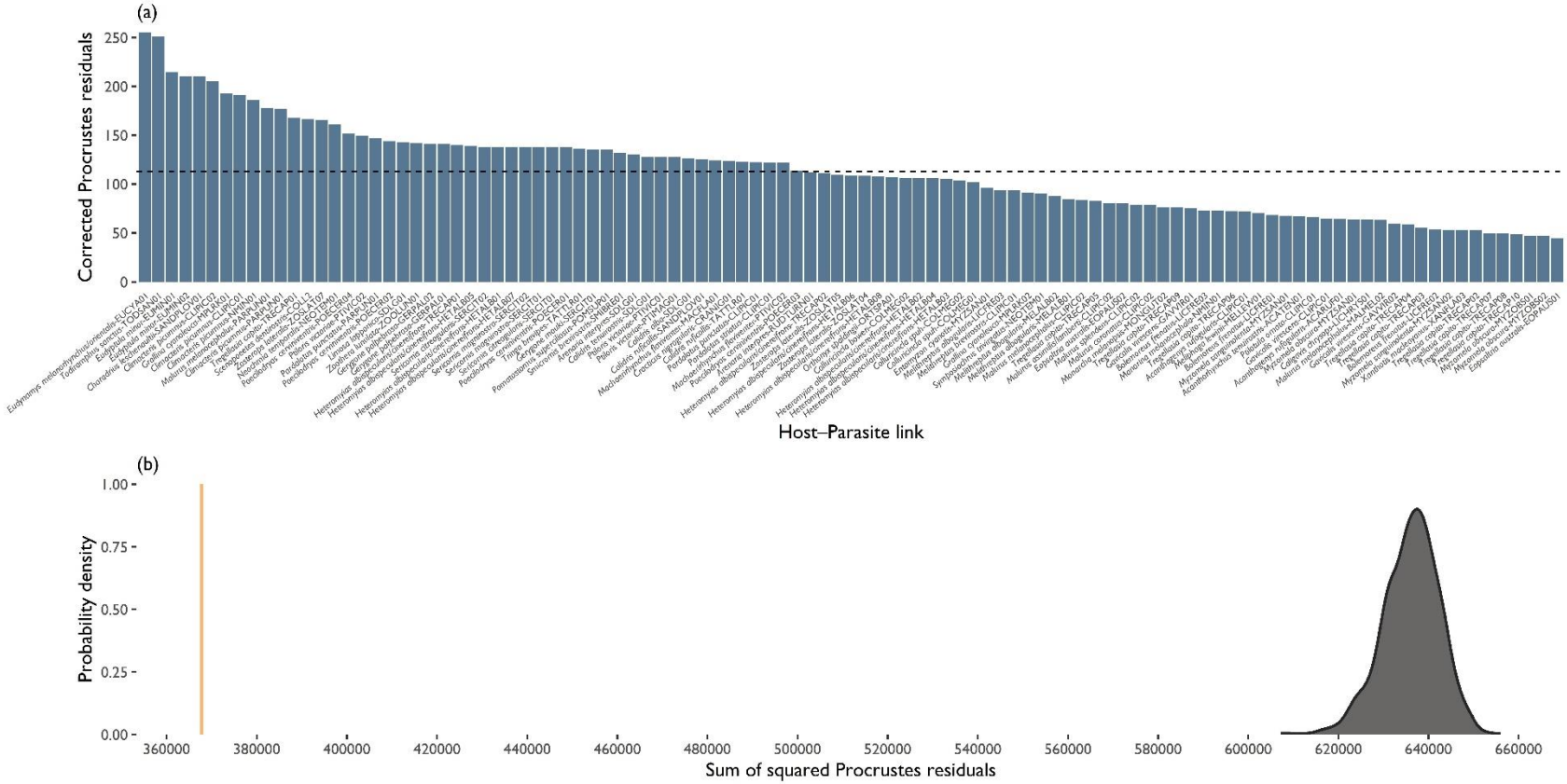

Figure S7. (a) Contributions of individual host–parasite linkages to the overall concordance between host and parasite phylogenies for all *Haemoproteus*–bird associations identified in Australia. Linkages with lower Procrustes residuals contribute relatively more to the concordance. The horizontal dashed line corresponds to the mean residual value across all linkages. **(b)** The overall congruence observed between the host and parasite phylogenies was significantly different from that expected under a null model in which the evolution of parasite lineages tracks that of host species and the cophylogenetic signal is determined by the degree of specialism or generalism of the parasite ($P = 0$).

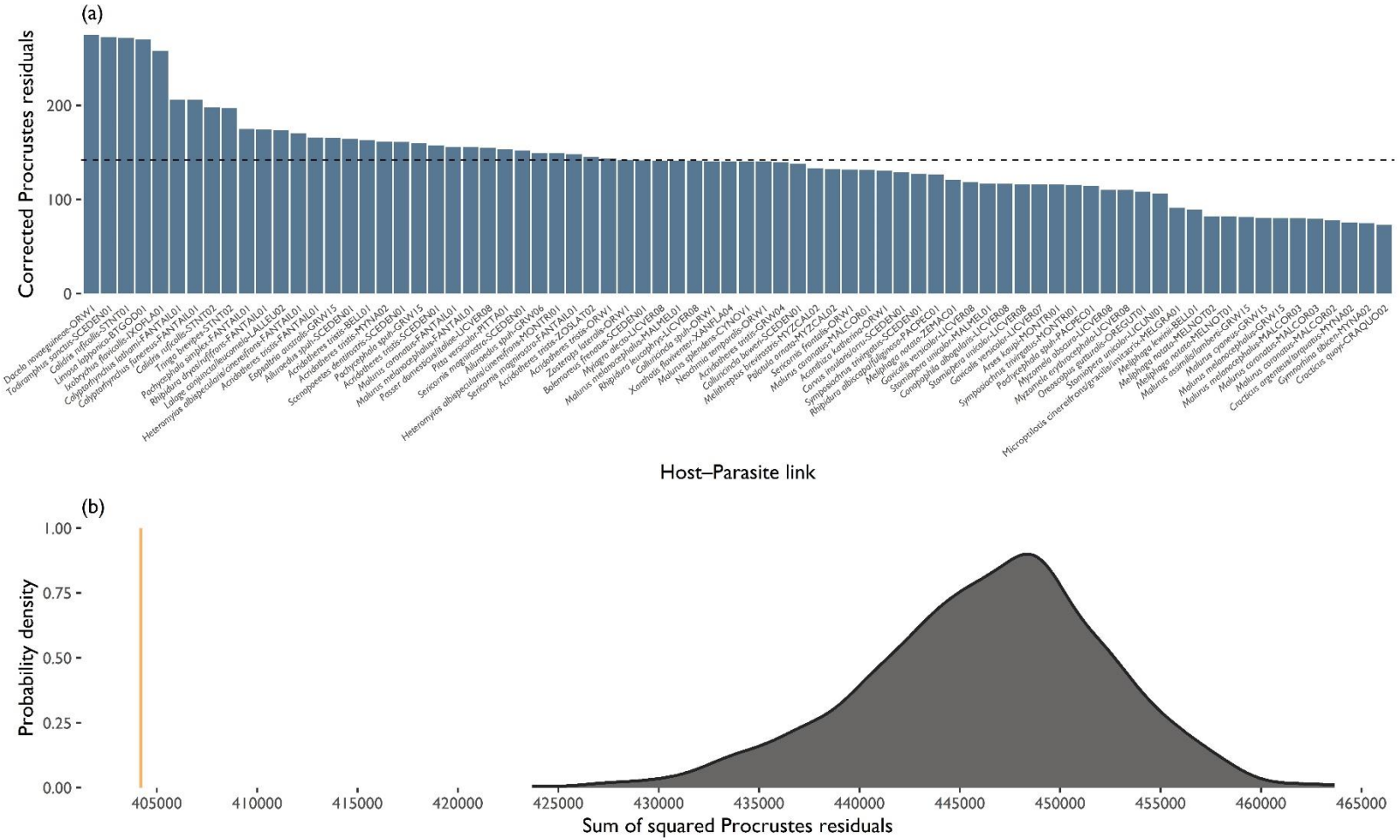

Figure S8. (a) Contributions of individual host–parasite linkages to the overall concordance between host and parasite phylogenies for all *Plasmodium*–bird associations identified in Australia. Linkages with lower Procrustes residuals contribute relatively more to the concordance. The horizontal dashed line corresponds to the mean residual value across all linkages. **(b)** The overall congruence observed between the host and parasite phylogenies was significantly different from that expected under a null model in which the evolution of parasite lineages tracks that of host species and the cophylogenetic signal is determined by the degree of specialism or generalism of the parasite ($P = 0$).

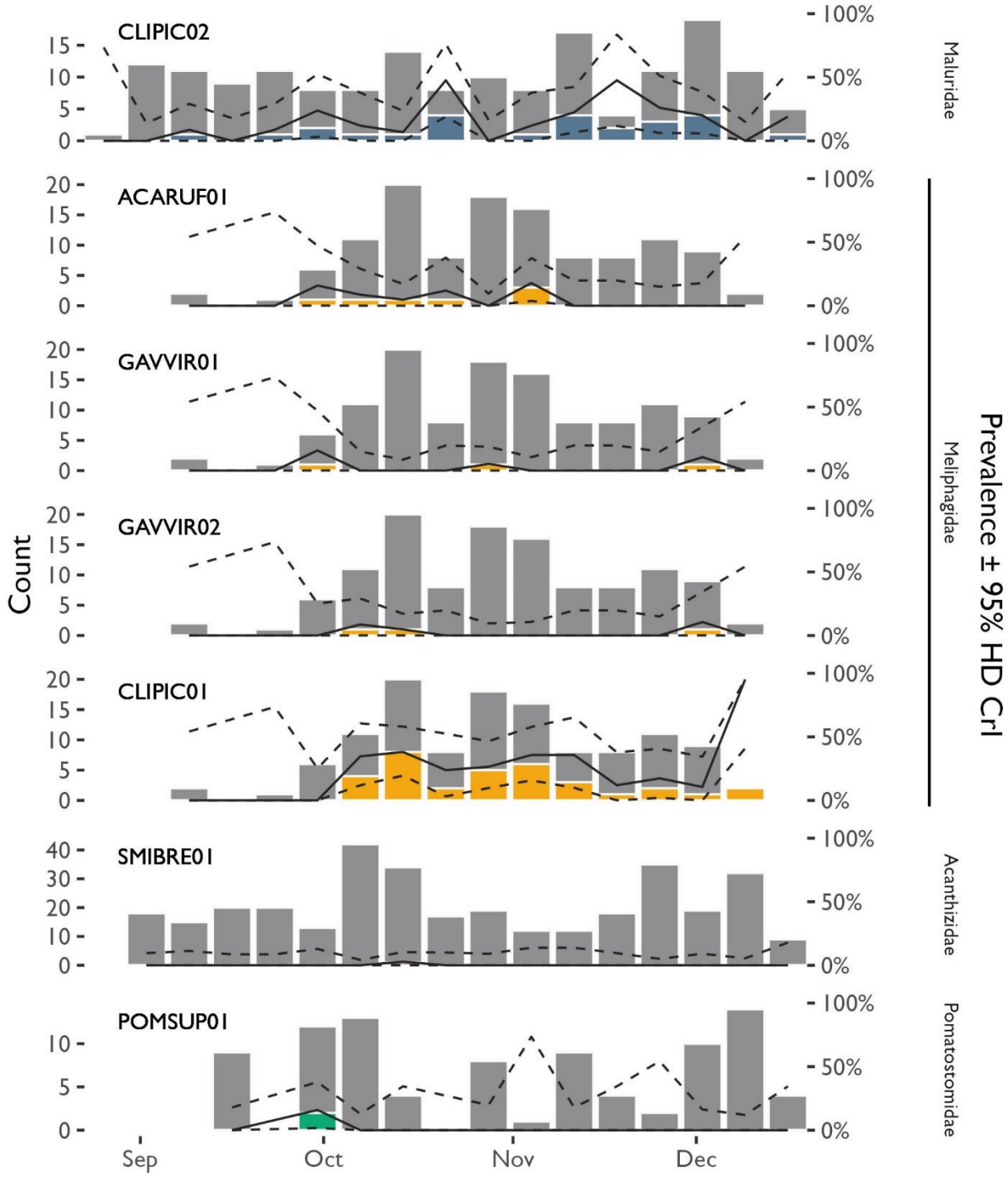

Figure S9. Time series of weekly sampling effort (number of individuals sampled, grey bars), *Haemoproteus*-positive birds (coloured bars; colour matches host family), and prevalence (solid lines). The 95% highest-density credible intervals (dashed lines) around the estimated prevalences were computed using Jeffreys priors (Beta(0.5,0.5)). Counts and prevalences are given separately for each *Haemoproteus* lineage and the host family in which each was identified. Only sampled adults are included.