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Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region

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Abstract

The degree to which widespread avian blood parasites in the genera *Plasmodium* and *Haemoproteus* pose a threat to novel hosts depends in part on the degree to which they are constrained to a particular host or host family. We examined the host distribution and host-specificity of these parasites in birds from two relatively understudied and isolated locations: Australia and Papua New Guinea. Using polymerase chain reaction (PCR), we detected infection in 69 of 105 species, representing 44% of individuals surveyed (n = 428). Across host families, prevalence of *Haemoproteus* ranged from 13% (Acanthizidae) to 56% (Petroicidae) while prevalence of *Plasmodium* ranged from 3% (Petroicidae) to 47% (Ptilonorhynchidae). We recovered 78 unique mitochondrial lineages from 155 sequences. Related lineages of *Haemoproteus* were more likely to derive from the same host family than predicted by chance at shallow (average LogDet genetic distance = 0, n = 12, P = 0.001) and greater depths (average distance = 0.014, n = 11, P < 0.001) within the parasite phylogeny. Within two major *Haemoproteus* subclades identified in a maximum likelihood phylogeny, host-specificity was evident up to parasite genetic distances of 0.029 and 0.007 based on logistic regression. We found no significant host relationship among lineages of *Plasmodium* by any method of analysis. These results support previous evidence of strong host-family specificity in *Haemoproteus* and suggest that lineages of *Plasmodium* are more likely to form evolutionarily–stable associations with novel hosts.

Keywords: Australia, avian malaria, *Haemoproteus*, host-specificity, Papua New Guinea, *Plasmodium*

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Introduction

The application of molecular methods to the study of avian haematozoa has revealed surprising levels of genetic diversity. This diversity has been exploited to reveal phylogenetic relationships (Perkins & Schall 2002), assess disease linkage between breeding and wintering grounds (Waldenström *et al*. 2002), and investigate host–parasite fidelity (Bensch *et al*. 2000; Ricklefs & Fallon 2002; Fallon *et al*. 2003). This last issue is of particular importance as human activities alter the ranges of vectors and avian hosts, thereby increasing exposure of potential hosts to novel parasites. In Hawaii, the introduction of the malarial parasite *Plasmodium relictum* has been implicated in the decline of native honeycreepers (van Riper *et al*. 1986). The negative impact of haematozoa introduced to domesticated birds has also been well documented (reviewed in Bennett *et al*. 1993a); however, discerning the fitness consequences of infections in wild birds with long histories of parasite exposure has been more difficult (Siikamaki *et al*. 1997; Hatchwell *et al*. 2001). Predicting the consequences of introduced disease is difficult, but we can begin to assess the chances of an exotic parasite spreading to novel hosts

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by determining the extent to which that parasite is evolutionarily constrained to a particular host or host family.

Two of the most common and best-studied genera of avian blood parasites are *Plasmodium* and *Haemoproteus*. Earlier studies have suggested that *Haemoproteus* exhibits greater host-specificity than *Plasmodium* (Bennett & Peirce 1988; Bennett *et al*. 1993b). Traditional means of classifying parasites at the species level, however, have often included host taxonomy as a character, thereby providing a biased estimate of host–parasite conservatism (Atkinson & van Riper 1991). In addition, reconstructions of parasite phylogenies based on DNA sequences have yielded evolutionary relationships that differ from those derived from traditional classification methods (Escalante *et al*. 1998).

A recent molecular study of *Haemoproteus* lineages in old world warblers and tits produced discordant host and parasite phylogenies, suggesting frequent host-switching (Bensch *et al*. 2000). A survey of parasites in African residents and European migrants revealed numerous cases of a single parasite lineage shared by multiple hosts; all *Haemoproteus* lineages were shared among hosts of the same family while at least one *Plasmodium* lineage occurred in multiple host families (Waldenström *et al*. 2002). On a global scale, Ricklefs & Fallon (2002) demonstrated relative conservatism of host–parasite evolution, but no distinction was made between the specificity of *Plasmodium* and *Haemoproteus*. Here, we attempt to merge the strengths of these studies by investigating host–parasite relationships at several evolutionary depths across multiple well-diversified host families within a single region.

As part of a global survey for the original host and geographical source of the Hawaiian parasite, we examined malarial parasites from a subset of bird species from tropical Australia and Papua New Guinea. To our knowledge, this is the first molecular exploration of host–parasite relationships in this fauna. The avifauna of this region is relatively isolated, both taxonomically and geographically, potentially reducing noise associated with transient introduction of foreign parasites. Prior surveys for haematozoan parasites have identified *Haemoproteus* and *Plasmodium* in many of the hosts included here, but relatively few parasites have been morphologically identified beyond the genus level (Ewers 1967; Bennett & Campbell 1973; Jones 1985). Our goals were to (1) characterize the prevalence of haematozoa across varied bird families in this region and (2) determine the extent to which *Haemoproteus* and *Plasmodium* differ in host-specificity.

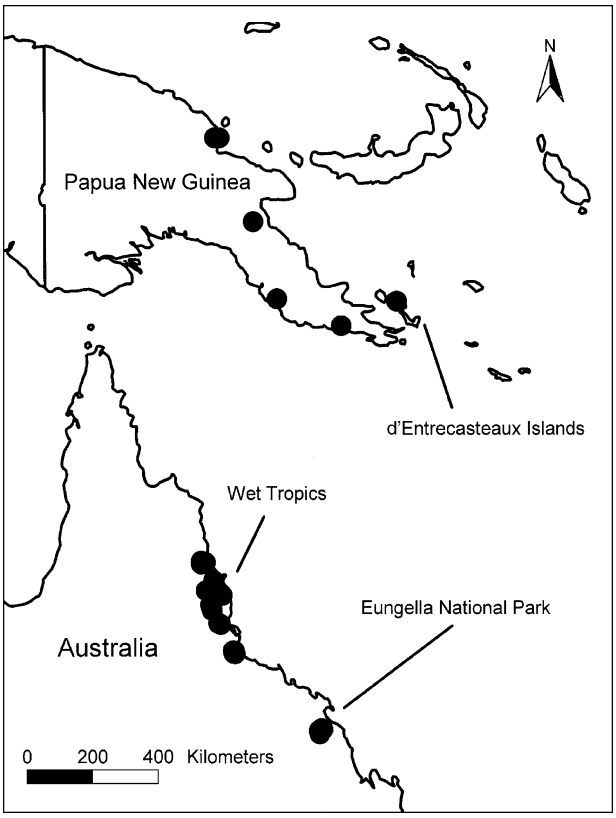

Fig. 1 Location of sampling sites in tropical Australia and Papua New Guinea.

Materials and methods

Sample collection and preparation

Blood samples were collected by J.A. from mist-netted birds in 2002 and 2003 at sites in the wet tropics of north-eastern Queensland, Australia and at Eungella National Park, which encompasses an isolated fragment of rainforest to the south (Fig. 1). Blood smears for 40 samples were fixed with methanol and then stained with Giemsa for 30 min. For each slide, we searched 100 fields at 400× magnification to determine infection status. High-resolution digital images of representative parasites were used for final identification.

Blood samples from birds captured in 2003 from the d'Entrecasteaux Islands, Papua New Guinea were provided by T.P. Blood and tissue samples of birds netted between 1991 and 2002 from forested sites across the main island of Papua New Guinea were provided by J.D.

We extracted host and parasite DNA from blood and tissue samples using the relevant protocols accompanying Qiagen DNeasy kits. Each extraction included a negative control, which was screened for contamination.

Parasite detection

In order to detect divergent and possibly degraded parasite DNA, we screened samples with two primer sets originally designed to successfully amplify *Haemoproteus* and *Plasmodium* DNA from dried blood smears up to 30 years old:

Table 1 Prevalence of *Plasmodium* and *Haemoproteus* assessed by polymerase chain reaction screening selected avian host families from the Australo-Papuan region

			Plasmodium		*Haemoproteus*		Genus unknown	
Host family	Species (*n*) screened	Samples (*n*) screened	Positive (*n*)	% Of total	Positive (*n*)	% Of total	Positive (*n*)	% Of total
Acanthizidae	12	69	9	13	9	13	1	2
Alcedinidae	8	23	1	4	5	22	1	4
Columbidae	8	17	1	6	9	53	1	6
Meliphagidae	15	70	7	10	34	49	1	1
Monarchidae	13	54	13	24	14	26	0	0
Pachycephalidae	17	94	9	10	20	21	7	7
Petroicidae	6	34	1	3	19	56	1	3
Ptilonorhynchidae	2	15	7	47	6	40	0	0
All families	80	376	48	13	116	31	12	3

Estimates of prevalence are biased low because identification of genus was not possible for all samples (Genus unknown).

850F (5′-CTT CAA CTA TTC TTA TAA AGT ATG T-3′) with 1024R (5′-AGG TGA GTG TTT TGC ATC ATT-3′) and F2 (5′-AAG TGA CCC AAC CTT AAA AAG-3′) with R2 (5′-GCT GTA TCA TAC CCT AAA GG-3′). Prior use of these primers in a wide array of avian hosts from varied geographical regions amplified no other haematozoa (e.g. *Leucocytozoon*, *Trypanosoma* and *Hepatozoon*). Primers 850F/1024R and F2/R2 amplify small fragments (167 bp and 132 bp) with homology to portions of mitochondrial cytochrome oxidase III and cytochrome *b* genes (Feagin 1992), respectively. We used annealing temperatures of 50 °C and 52 °C, respectively, and typical PCR reactions employed conditions developed for amplification of 'ancient' DNA (Fleischer *et al*. 2000).

For those samples that were positive based on the tests above, we amplified a larger fragment of cytochrome *b* (533 bp + primers) for use in phylogenetic analyses using primers 3760F (5′-GAG TGG ATG GTG TTT TAG AT-3′) and 4292Rw2 (5′-TGG AAC AAT ATG TAR AGG AGT-3′). If this fragment did not amplify, we attempted to amplify smaller fragments of either 433 bp or 295 bp (+ primers) using either F1 (5′-CAT ATT TAC CTT TAT CAT GGA T-3′) or F3 (5′-CCA GGA CTT GTT TCA TGG AT-3′) with 4292Rw2. The annealing temperature for these last reactions was 51 °C.

To ensure that DNA extractions were successful for those samples in which we did not detect infection, we amplified a small fragment (268 bp) of avian cytochrome *b* DNA using primers cytb-2RC and cytb-wow following the methods described in Dumbacher *et al*. (2003). This amplification was successful in all cases.

Following purification of PCR products using Qiaquick kits (Qiagen), we bidirectionally sequenced the largest fragment available for a given sample on an ABI 3100 Sequencer (Applied Biosystems). Sequences were assembled, aligned and edited using the program SEQUENCHER version 4.1. Phylogenies based on cytochrome *b* sequence have consistently recovered two discrete clusters of lineages corresponding to *Haemoproteus* and *Plasmodium* (Bensch *et al*. 2000; Perkins & Schall 2002). Therefore, we assigned mitochondrial sequences (lineages) to each genus based on their associations in a phylogenetic tree (see below). Inclusion of sequence data from prior studies and morphological assessments of parasites for which we had smears generally allowed easy delineation of the two genera. In cases where limited sequence data did not provide sufficient resolution, we used a restriction enzyme test (J.S.B. and R.C.F. unpublished) to assign parasite lineages to genera.

To assess whether prevalence of *Haemoproteus* and *Plasmodium* varied across host families, we performed an ANOVA (GLM in SAS version 8.2; SAS Institute) on arcsine square-root transformed prevalences observed at the level of host species. We included only those species from families represented by greater than 10 individuals total (Table 1). We estimated the proportion of variance attributable to host family using the NESTED procedure in SAS.

Cloning

In several cases, we detected multiple infections based on the occurrence of multiple peaks throughout the chromatogram. In these cases, we repeated the polymerase chain reaction (PCR) and cloned the fragment using a TOPO-TA cloning kit (Invitrogen) following manufacturer guidelines. We picked 6–24 blue/white-selected colonies for each fragment cloned, boiled the colonies for 10 min, and amplified 2 μL of the resulting lysate for 30 cycles with the relevant primer set. Fragments from successful amplifications were cleaned and sequenced as described above. Inspection of sequences obtained for a given clone, and comparison of

those sequences with the original sequence, allowed for easy identification of PCR artefacts arising from polymerase error or *in vitro* recombination (Thompson *et al*. 2002).

Phylogenetic analysis

We estimated parasite phylogenetic relationships using all samples for which we had at least 295 base pairs of cytochrome *b* sequence, although 533 bp were available for most samples (see Appendix I). Following the phylogeny developed by Perkins & Schall (2002), all trees were rooted with mammalian *Plasmodium* sequences (GenBank accession nos. AY069614, AF069624, AF055587, AY099051, AY283019 and AF069610). The program MODELTEST version 3.06 (Posada & Crandall 1998) indicated that the most likely model of base pair substitution was general time reversible (GTR), with the proportion of invariable sites = 0.3604 and gamma shape parameter = 0.5372. We used maximum likelihood (ML) to reconstruct a phylogeny using these parameters. We used 100 replicates and the 'fast' heuristic in PAUP* (Swofford 1999) to estimate bootstrap support. We also performed a full heuristic search for the shortest tree using tree-bisection reconnection (TBR) on both GTR and LogDet (Lockhart *et al*. 1994) distances. We compared the resulting minimum evolution tree to 1000 trees generated by bootstrap resampling with a TBR heuristic search. Nodes with greater than 50% support were retained.

Host-specificity

We followed the binomial probability approach of Ricklefs & Fallon (2002) to assess the extent to which parasites of varying relatedness were likely to be found in host species from the same family. Host species were grouped into families as listed in the *Handbook of the Birds of the World* (del Hoyo *et al.* 2003), but we grouped all kingfishers in the Alcedinidae and included *Rhipidura* fantails within the Monarchidae (Sibley & Ahlquist 1985). First, we tested for a significant difference between the observed and expected probability that a shared parasite lineage (i.e. mitochondrial haplotypes indicated by light blue dots in Fig. 2) derived from two host species of the same family. We calculated this separately for shared *Haemoproteus* and *Plasmodium* lineages. In cases where a single parasite lineage was found in more than two host species, we randomly paired hosts to represent that lineage. For example, if a lineage occurred in six different hosts, we randomly paired those hosts to form three observations.

Subsequently, we repeated the analysis using pairs of parasite lineages joined by first-step nodes with greater than 70% bootstrap support (dark blue dots in Fig. 2). When a first-step node joined more than two host taxa, we randomly chose just a single independent pair. To quantify the phylogenetic depth being analysed, we calculated average pairwise LogDet distances among parasites compared at each level. For all comparisons we used only lineages with greater than 470 bp of sequence.

In order to extend the analysis beyond first-step nodes and to assess the parasite genetic distance at which host family conservatism was lost, we performed a logistic regression of host family (same or different) vs. LogDet parasite distance (Ricklefs & Fallon 2002). We tested for a significant influence of region (same or different) on host family similarity before using the full data set for each application of the model. Logistic regression employs the model $\ln(P/1-P) = a + b*d$ where P is the probability that two parasites derive from hosts of the same family, d is genetic distance, and a and b are coefficients estimated by the model. We performed this regression on all pairwise comparisons of parasite lineages and their hosts at several levels of evolutionary organization. Because multiple pairwise distance comparisons violate assumptions of independence, we determined significance of the coefficients using a permutation of the original data. We randomly reassigned host families to the parasite phylogeny 999 times and performed logistic regression upon each iteration. Coefficients based on the original data were compared with those generated by randomization in order to estimate the probability of recovering the original estimates by chance alone.

Results

Parasite prevalence

We used PCR to screen 428 individuals in total. Of 209 individuals from Papua New Guinea, 64 (31%) tested positive for *Haemoproteus* and 20 (10%) tested positive for *Plasmodium*. Of 77 species tested, 46 were positive for one or both genera and we detected infection in all 12 species for which we tested five or more individuals. Of 219 individuals tested from Australia, 62 (28%) were positive for *Haemoproteus* and 30 (14%) were positive for *Plasmodium*. We recovered *Haemoproteus* or *Plasmodium* from 27 of 32 species tested, and we found infection in 17 of 19 species for which five or more individuals were screened. A χ^2 test revealed no significant difference in prevalence of either parasite between regions.

Low PCR amplification, poor-quality sequence or unresolved multiple infections reduced the number of samples for which we could identify parasites to genus, and therefore, estimates of prevalence (Table 1) were biased low. Prevalence of *Haemoproteus*, which ranged from 13% in the Acanthizidae to 56% in the Petroicidae was not uniform across different host families ($F = 3.71$, df = 7, $P = 0.002$), however, host family grouping explained only 22% of the total variance in prevalence among different host species. Except in the Ptilonorhynchidae, prevalence of *Plasmodium* was relatively low, and no significant difference was

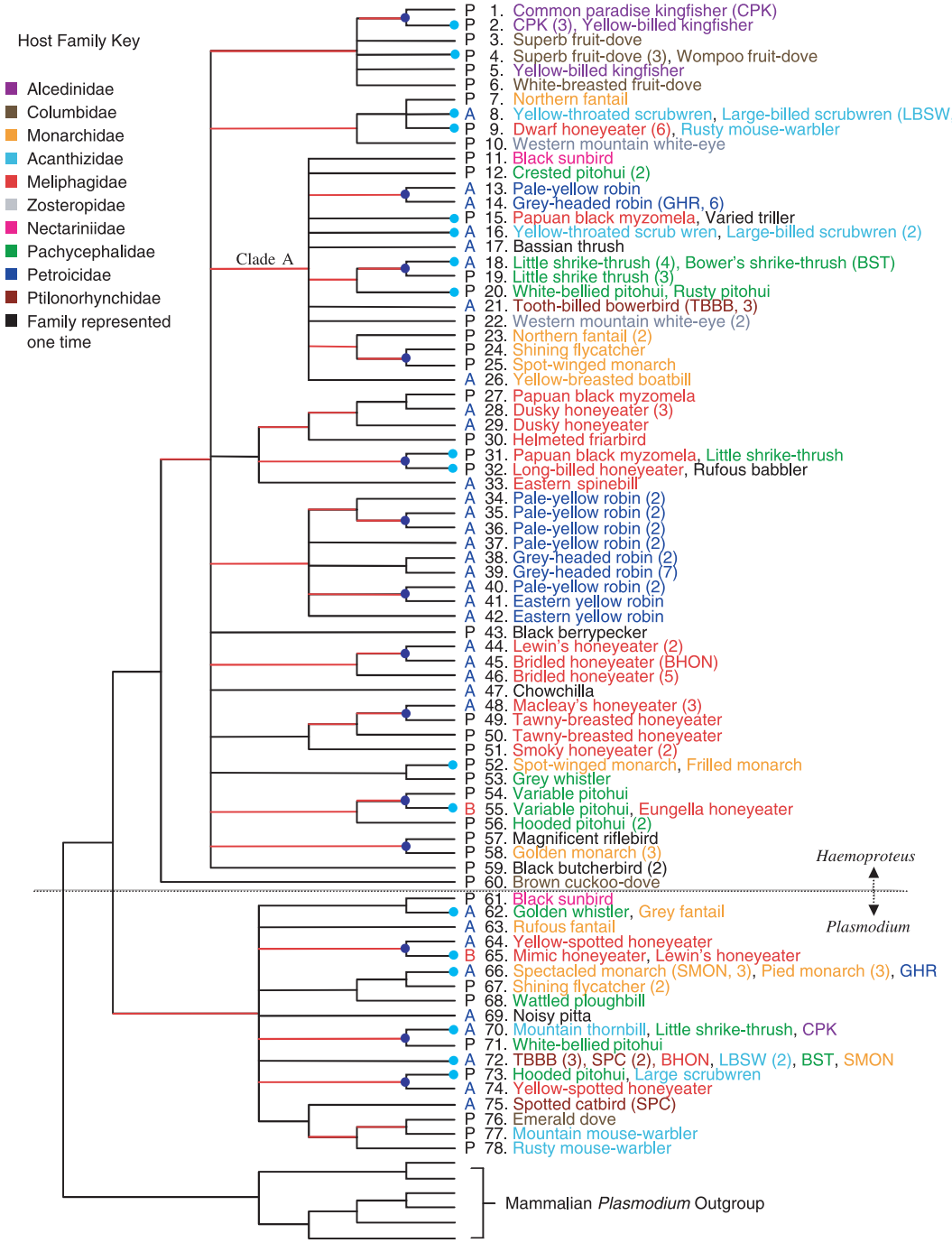

Fig. 2 Cladogram depiction of neighbour-joining tree based on LogDet distances between mitochondrial lineages of avian haematozoa. Region of origin (A for Australia, P for Papua New Guinea, B for both regions), lineage number, host species (colour-coded for family), and frequency of detection (number in parenthesis when recovered more than once) are indicated at right. Red branches indicate bootstrap support greater than 70% (1000 replicates). Pale blue and dark blue dots indicate lineages used for binomial tests of host conservation.

evident among families ($F = 1.39$, df = 7, $P = 0.223$). Only about 4% of the variance in prevalence between species could be attributed to host family. Family assignment and frequency of parasite detection for all host species examined is listed in Appendix II.

We detected mixed infections in 29 individuals. Among those with enough sequence data to identify parasite genera present, one individual harboured two *Plasmodium* lineages (66 and 72), 11 harboured two *Haemoproteus* lineages (see below) and four harboured mixed *Plasmodium*/

Haemoproteus infection (11 and 61, 16 and 72, 21 and 72, 18 and 70). Of the lineages involved in mixed *Haemoproteus* infections, four pairs derived from within well-supported clades composed of nonpasserines (3 and 4), Meliphagidae (28 and 29), or Petroicidae (35 and 36, 35 and 37). The remaining pairs (10 and 22, 13 and 37, 14 and 38, 14 and 39 repeatedly) were composed of parasites from each of the two main subclades (see phylogenetic results below). The average LogDet genetic distance between parasite combinations was 0.0623 ($n = 1$) for mixed *Plasmodium*, 0.0414 ($n = 11$) for mixed *Haemoproteus* and 0.1352 ($n = 4$) for mixed *Haemoproteus*/*Plasmodium*.

Reliability of methods

Failure to detect infection by PCR may have been due to low-quality or insufficient template, small daily variation in PCR conditions and reaction composition, and mismatches between the primer and parasite DNA template. To generate a minimum estimate of our detection error, we divided the number of false negatives produced by a given primer set by the total number of samples that were known to be positive by either primer set. By this method, the primer set F2/R2 had an error rate of 30%, while primer set 850F/1024R missed infections at a rate of 17%. Therefore, even under favourable PCR conditions, the chance that both primer sets failed to detect an infection was about 5%.

Estimation of haematozoa presence/absence was identical for 35 of 40 samples analysed by both PCR and visual inspection of blood smears. The PCR screening detected infection in three samples that went undetected by examination of blood smears. Conversely, an initial inspection of blood smears suggested that PCR had missed infections in two samples. Subsequent scanning of the slides by an unbiased second observer (M.P.), however, suggested that artefacts in these two slides had been misidentified as parasites. In samples where both methods identified a parasite to genus, seven of eight matched. The single disparity in genus identification was attributed to a poorly prepared slide and a second appraisal of the slide suggested that the parasite was representative of either *Haemoproteus* or *Plasmodium*. No other haematozoa were observed in blood smears.

Phylogenetics

Among the 165 samples for which we had at least 295 bp of sequence, we found 78 unique mitochondrial lineages: 60 *Haemoproteus* and 18 *Plasmodium* (GenBank accession numbers are listed in Appendix I). Lineage 60, isolated from *Macropygia amboienensis*, was included with *Haemoproteus* based on evidence from the restriction assay and morphological assessment of a parasite with a closely related mtDNA sequence (E.G. unpublished). Related lineages have also been found in *Columbina passerina* from North America (unpublished data) and other doves (S Fallon personal communication). Phylogenies developed using ML and LogDet and GTR distances were similar. Because each of these methods yielded similar topologies and for consistency with previous work by Ricklefs & Fallon (2002), we used a tree derived from LogDet distances for tests of host-parasite specificity (Fig. 2).

Within *Haemoproteus*, our data could not resolve deep hierarchical relationships, which resulted in a large basal polytomy. Parasites from two nonpasserine host families occurred in a unique, well-supported clade (top of Fig. 2). Other clades descending from the genus-level polytomy included several which were largely derived from a single host family (Meliphagidae, Petroicidae or Pachycephalidae) and one well-supported clade with diverse host family representation (clade A). Several well-supported host-family specific clades (Petroicidae, Pachycephalidae and Monarchidae) were nested within clade A. An ML estimate of the phylogeny (Fig. 3) identified three major clades within *Haemoproteus*: two lineages derived from passerine hosts (clades A and B) and a third composed of lineages from the two nonpasserine families studied. Bootstrap support was relatively low for all but the nonpasserine clade. The ML phylogeny also indicated monophyly of all unshared parasites recovered from Meliphagidae.

Deeper level relationships among *Plasmodium* lineages were similarly unresolved in a distance-based phylogeny (Fig. 2). Beneath the genus-level polytomy, only a pair of lineages (64 and 65) from Meliphagidae fell into a small well-supported host-specific clade.

Host specificity

We found 12 *Haemoproteus* lineages that were each shared by two different host species and we found six *Plasmodium* lineages in more than one host species. Three of these *Plasmodium* lineages were each found in three to six host species. Related lineages of *Haemoproteus* were more likely to be found in related hosts than predicted by chance. At average parasite genetic distances of 0 (shared identical lineages) and 0.014 (1st-step nodes), the probability of related parasites deriving from the same host family was 0.58 ($n = 12$, $P = 0.001$) and 0.73 ($n = 11$, $P < 0.001$), respectively.

Sample sizes for comparisons within the *Plasmodium* genus were smaller. The probability that a shared *Plasmodium* lineage derived from the same host was 0.13 (average distance = 0, $n = 8$, $P = 0.65$). This value was not significant even if pairs of hosts were chosen so as to maximize the probability (probability = 0.38, $n = 8$, $P = 0.11$). Similarly, sister lineages joined by first-step nodes were not significantly likely to have derived from the same host family (average distance = 0.008, probability = 0.33, $n = 3$, $P = 0.61$).

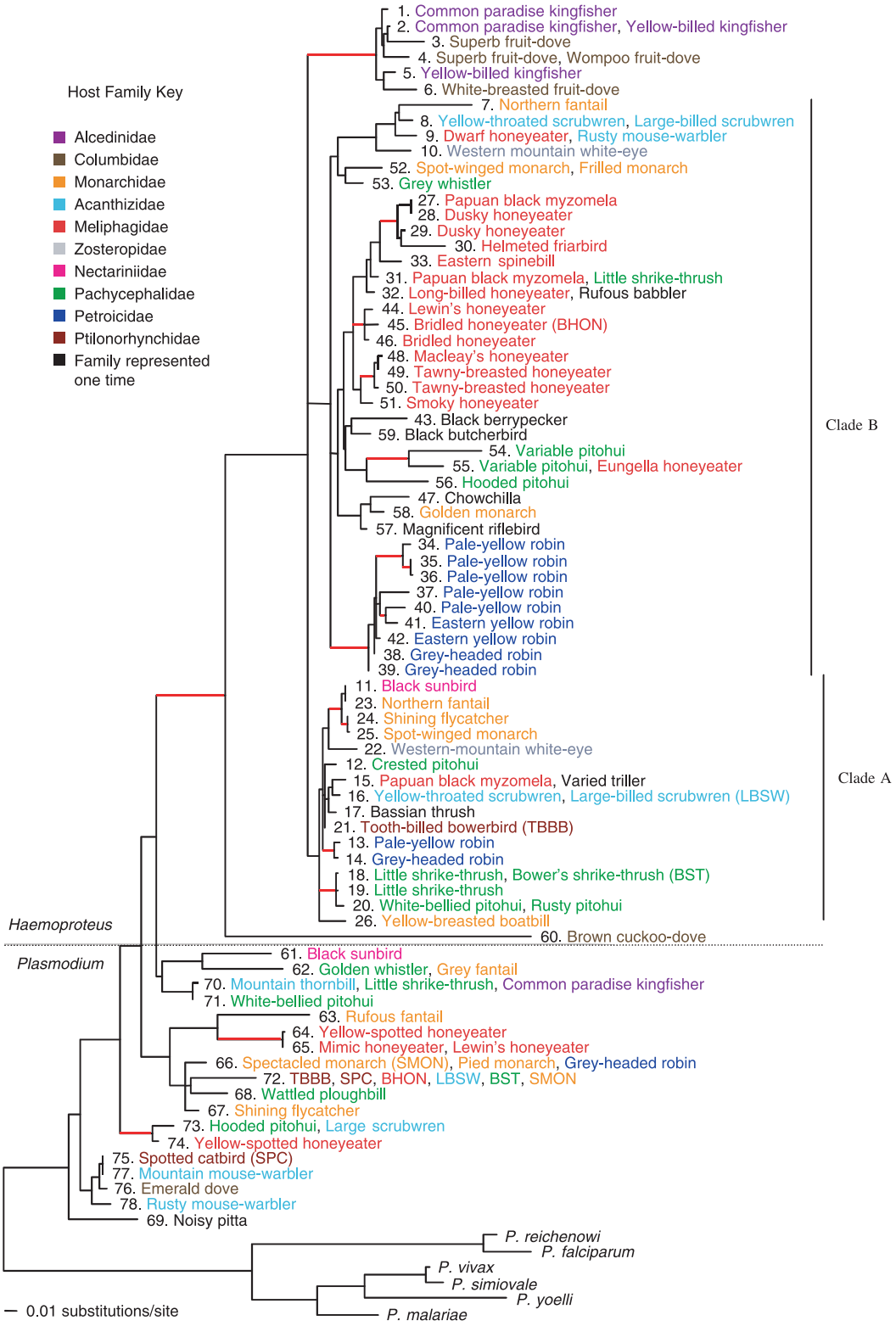

Fig. 3 Relationships among haematozoan parasites based on maximum likelihood using the model GTR + I + G. Lineage number and host species (colour-coded for family) are indicated at right. Red branches indicate bootstrap support greater than 70% (stepwise addition, 100 replicates).

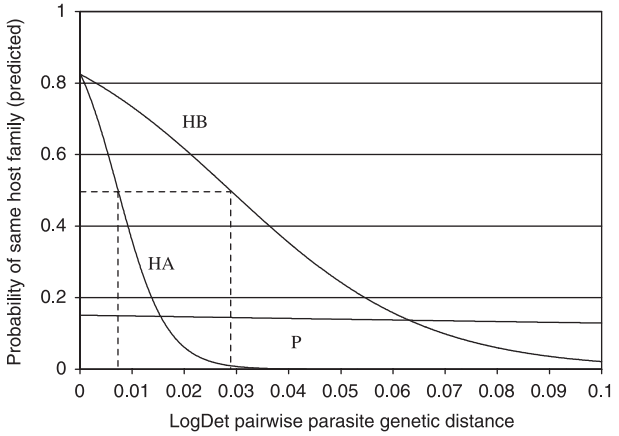

Fig. 4 Logistic regression curves relating the predicted probability of host relatedness to genetic differentiation of parasites in the genus *Plasmodium* (P) and *Haemoproteus* clade A (HA) and clade B (HB). Dotted lines indicate the genetic distance at which parasite pairs from clades A and B were equally likely to be found in hosts of the same or different families.

We applied logistic regression to four groups of parasites: all *Plasmodium* lineages, all *Haemoproteus* lineages, *Haemoproteus* clade A and *Haemoproteus* clade B. Because 'region' did not contribute significantly to the regression of host family on distance, we considered Australia/Papua New Guinea to be one region for all logistic regression analyses. Regression coefficients for the genus *Haemoproteus* were significant ($a = 0.1909$, $b = -38.57$, $P < 0.001$) as were coefficients for clade A ($a = 1.5701$, $b = -214.6$, $P < 0.001$) and clade B ($a = 1.5513$, $b = -53.7664$, $P < 0.001$). Coefficients for the genus *Plasmodium* were not significant ($a = -1.7248$, $b = -1.8373$, $P = 0.63$). By evaluating the regression equation at $P = 0.5$, we could estimate the genetic distance at which pairs of parasite lineages were equally likely to have derived from the same or different host family. This distance at which host-family signal was lost was 0.005 for all *Haemoproteus* lineages, 0.007 for clade A, and 0.029 for clade B. Evaluating the regression equation at a distance of zero, the predicted probability of finding identical parasites in hosts of the same family was 0.55 evaluated over all *Haemoproteus* lineages, 0.83 for clade A and 0.83 for clade B. Figure 4 depicts the predicted regression curves for *Plasmodium* and *Haemoproteus* clades A and B.

Discussion

Epizootiology

Blood parasites in the genera *Haemoproteus* and *Plasmodium* appear to be nearly ubiquitous in avian communities. We detected one or both of these genera in almost 66% of species and this number would likely rise substantially with deeper sampling of individual species. In the Australo-Papuan region studied, we estimated an overall prevalence of about 44% with no significant differences between north-east Australia and New Guinea lowlands. Estimates of prevalence in tropical regions have ranged from about 10% in Costa Rica and the neotropics (White *et al*. 1978; Young *et al*. 1993; by blood smear) to 28% in the Lesser Antilles (S. Fallon personal communication), 40% in Central Africa (Richard *et al*. 2002) and 59% in American Samoa (*Plasmodium* only, Jarvi *et al*. 2003). Comparison of prevalence across surveys is confounded by differences in sensitivity of the diagnostics employed (Richard *et al*. 2002), and our PCR technique underestimated infection by at least 5%. Serological tests may provide the most accurate estimate of infection by detecting low-level chronic infections (Jarvi *et al*. 2002), but interpretation of the assays can be difficult (Jarvi *et al*. 2003), lineage identification is impossible, and the methods may not be applicable across varied hosts and parasite lineages.

Comparison of prevalence among regions is also likely to be confounded by the host families sampled. Except in the *Ptilonorhynchidae*, which were sampled only sparsely, prevalence of *Plasmodium* was low and fairly uniform among well-represented host families. On the other hand, prevalence of *Haemoproteus* varied significantly among host families, and this could bias regional comparisons in cases where families are not represented equally. Although certain host families such as the Columbidae repeatedly exhibit relatively high prevalence of infection across studies (Atkinson & van Riper 1991), estimates of prevalence, even if accurate, should be considered snapshots in time and host space (Bensch & Akesson 2003; Scheuerlein & Ricklefs 2004). Infection rates can vary dramatically between years and may be more representative of differences in vector abundance and their distribution within different habitats than family level differences in host immune response or other evolved characters (Bennett & Cameron 1974).

Within the Pachycephalidae, we expected that parasite prevalence might have been lower among pitohuis, a group which produces varying amounts of a toxic alkaloid potentially active in invertebrate vectors (Dumbacher *et al*. 1992). The overall infection rate in this group (40%), however, was close to both the value for the entire family (35%) and the average prevalence within Papua New Guinea (46%). This suggests little role for the toxin in vector deterrence, however, collection of Papuan birds occurred over several years and the caveats mentioned above may apply.

We uncovered multiple infections from a wide range of hosts. Given that at least 40% of individuals were infected by either *Haemoproteus* or *Plasmodium*, the prevalence of mixed infections should have been fairly high if not constrained by parasite–parasite interaction (Hatchwell *et al*. 2000). The 29 cases of multiple infection that we uncovered fell below the expected number of about 60 (based on

overall prevalences of about 30% and 12% for *Haemoproteus* and *Plasmodium*, respectively). While this may be indicative of competitive exclusion, the cases of multiple infection observed represent a minimum since we did not recover sequence data from every infected individual and even successful PCR was likely to miss some multiple infections owing to primer bias or unequal quantities of parasite DNA. Haematozoan genera may have evolved distinctive antigenic signatures that avoid cross-generic immunity in a common host (Atkinson & van Riper 1991), but the extent to which the evolutionary relatedness of parasites within genera influences interlineage competition and thus, the distribution of parasites, should be addressed more carefully in the future.

Host–parasite evolution

Parasite lineages found in more than one host have often been cited as evidence of host-switching. While the introduction of parasites into novel hosts is a prerequisite for host-switching, the current distribution of parasites may not reflect long-term coevolution between the parasite and its vertebrate host, but may be more indicative of the cosmopolitan feeding of its invertebrate vector. Generalist feeders such as mosquitoes or ceratopogonid flies may drive the continuous introduction of varied *Plasmodium* and *Haemoproteus* lineages into diverse hosts. Not all of these interactions will necessarily be stable throughout time. For example, Atkinson (1986) demonstrated that *Haemoproteus meleagridis*, a parasite commonly found in turkeys, was capable of developing in other Galliformes, but infections were transient and rapidly cleared from these secondary hosts to which the parasite may have been poorly adapted.

We found several lineages of both *Plasmodium* and *Haemoproteus* in multiple host families, however, identical *Haemoproteus* lineages were more likely to derive from related hosts than *Plasmodium*. Even if we assume that these cases represent evolutionarily stable changes in host affinity, recent host-switching by *Haemoproteus* lineages has been relatively constrained to related hosts. The significant signal of host family specificity observed in *Haemoproteus* at greater depths within the phylogeny, however, suggests that not all of the apparent associations between a single parasite lineage and multiple host families represent stable interactions. Given the host-family conservatism at first-step nodes and the strong signals from logistic regression, evolutionarily stable jumps between host families are likely to be rare in the genus *Haemoproteus*.

Across the genus *Haemoproteus*, the signal for host family specificity was lost at a parasite divergence of about 0.005. The attenuation in the signal, measured across the entire genus, was probably due to the structure of relationships between lineages within the genus. Analysed separately, the two large subclades of *Haemoproteus* lineages derived from passerine hosts both exhibited strong host specificity. For clade B, in which the average pairwise divergence among parasites was about 0.075, the host signal extended to a parasite divergence of about 0.029. Within clade A, average pairwise parasite divergence was only about 0.021, and host-specificity was evident up to a parasite divergence of only 0.007.

Lineages within clades A and B may have diversified via periodic host-switching following an early vicariance event in an ancient *Haemoproteus* lineage. In both clades, however, we were largely unable to recover well-supported hierarchical relationships among groups of parasites derived from different host families, suggesting that the common ancestor to each clade spread rapidly across host families. Assuming that rates of nucleotide substitution are similar across various lineages of *Haemoproteus*, the short branch lengths in clade A suggest a relatively recent radiation of parasites across host taxa. Without further sampling, it will remain unclear how frequently lineages have escaped otherwise strong host constraint. If younger parasite radiations have spread broadly across avian hosts in the past, this phenomenon of escape and radiation would continually reset the parasite molecular clock relative to the avian clock. This in turn could help to explain the apparent slow divergence of parasite DNA relative to host DNA noted by Ricklefs & Fallon (2002).

Parasites in the genus *Plasmodium* appeared to be less constrained by the phylogenetic relationships of their hosts and showed no evidence of host-specificity at any depth within the parasite phylogeny. Our relatively small sample of *Plasmodium* may have limited our power to detect a signal, however, we detected host-specificity within the equally small *Haemoproteus* clade A. Interestingly, Ricklefs & Fallon (2002) detected host conservatism across both *Haemoproteus* and *Plasmodium* up to a parasite divergence of 0.026. Because they applied logistic regression across lineages from both genera, however, it is unclear how that value partitioned between genera or between distinct radiations within genera. The evidence here supports a broad host range for at least some *Plasmodium* parasites and indicates a tendency for a high level of evolutionarily stable host-switching. Of the two parasite genera studied, *Plasmodium* likely presents the greatest threat of colonizing novel hosts and may warrant the most attention when managing the welfare of isolated and naive hosts.

Most of the avian lineages sampled for parasites derived from a radiation of songbirds unique to the Australo-Papuan region (Sibley & Ahlquist 1985). In addition, birds from tropical Australia and New Guinea may be more isolated than their continental counterparts such that interactions between hosts, vectors and parasites that would otherwise confound estimates of host-specificity are minimized. Nonetheless, trends in host-specificity observed in the Australo-Papuan region appear to be in line with the picture emerging from many other regional studies

(partial summary in Schrenzel *et al*. 2003). Additional molecular surveys of parasites at the regional level will add further insight into patterns of host–parasite interaction.

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Appendix I

Avian host names, geographical origin, sequence length and GenBank accession numbers for parasite lineages listed in Fig. 2

Lineage	Host information Family	Genus	Species	Common name	Locality	bp	GenBank Acc. No.
1	Alcedinidae	*Tanysiptera*	*galatea*	Common paradise kingfisher	PNG	487	AY714134
2	Alcedinidae	*Tanysiptera*	*galatea*	Common paradise kingfisher	PNG	533	AY714135
2	Alcedinidae	*Halcyon*	*torotoro*	Yellow-billed kingfisher	PNG	533	AY714135
3	Columbidae	*Ptilinopus*	*superbus*	Superb fruit-dove	PNG	533	AY714136
4	Columbidae	*Ptilinopus*	*superbus*	Superb fruit-dove	PNG	533	AY714137
4	Columbidae	*Ptilinopus*	*magnificus*	Wompoo fruit-dove	PNG	533	AY714137
5	Alcedinidae	*Halcyon*	*torotoro*	Yellow-billed kingfisher	PNG	533	AY714138
6	Columbidae	*Ptilinopus*	*rivoli*	White-breasted fruit-dove	PNG	533	AY714139
7	Monarchidae	*Rhipidura*	*rufiventris*	Northern fantail	PNG	295	AY714140
8	Acanthizidae	*Sericornis*	*citreogularis*	Yellow-throated scrubwren	AUS	533	AY714141
8	Acanthizidae	*Sericornis*	*magnirostris*	Large-billed scrubwren	AUS	533	AY714141
9	Meliphagidae	*Oedistoma*	*iliolophus*	Dwarf honeyeater	PNG	533	AY714142
9	Acanthizidae	*Crateroscelis*	*murina*	Rusty mouse-warbler	PNG	533	AY714142
10	Zosteropidae	*Zosterops*	*fuscicapillus*	Western mountain white-eye	PNG	533	AY714143
11	Nectariniidae	*Nectarinia*	*aspasia*	Black sunbird	PNG	295	AY714144
12	Pachycephalidae	*Pitohui*	*cristatus*	Crested pitohui	PNG	533	AY714145
13	Petroicidae	*Tregellasia*	*capito*	Pale-yellow robin	AUS	533	AY714146
14	Petroicidae	*Heteromyias*	*albispecularis*	Grey-headed robin	AUS	533	AY714147
15	Campephagidae	*Lalage*	*leucomela*	Varied triller	PNG	533	AY714148
15	Meliphagidae	*Myzomela*	*nigrita*	Papuan black myzomela	PNG	533	AY714148
16	Acanthizidae	*Sericornis*	*citreogularis*	Yellow-throated scrubwren	AUS	533	AY714149
16	Acanthizidae	*Sericornis*	*magnirostris*	Large-billed scrubwren	AUS	533	AY714149
17	Muscicapidae	*Zoothera*	*lunulata*	Bassian thrush	AUS	533	AY714150
18	Pachycephalidae	*Colluricincla*	*megarhyncha*	Little shrike-thrush	AUS	533	AY714151
18	Pachycephalidae	*Colluricincla*	*boweri*	Bower's shrike-thrush	AUS	533	AY714151
19	Pachycephalidae	*Colluricincla*	*megarhyncha*	Little shrike-thrush	AUS	533	AY714152
20	Pachycephalidae	*Pitohui*	*incertus*	White-bellied pitohui	PNG	533	AY714153
20	Pachycephalidae	*Pitohui*	*ferrugineus*	Rusty pitohui	PNG	533	AY714153
21	Ptilonorhynchidae	*Scenopoeetes*	*dentirostris*	Tooth-billed bowerbird	AUS	533	AY714154
22	Zosteropidae	*Zosterops*	*fuscicapillus*	Western mountain white-eye	PNG	533	AY714155
23	Monarchidae	*Rhipidura*	*rufiventris*	Northern fantail	PNG	533	AY714156
24	Monarchidae	*Myiagra*	*alecto*	Shining flycatcher	PNG	533	AY714157
25	Monarchidae	*Monarcha*	*guttula*	Spot-winged monarch	PNG	533	AY714158
26	Monarchidae	*Machaerirhynchus*	*flaviventer*	Yellow-breasted boatbill	AUS	271	AY714159
27	Meliphagidae	*Myzomela*	*nigrita*	Papuan black myzomela	PNG	533	AY714160
28	Meliphagidae	*Myzomela*	*obscura*	Dusky honeyeater	AUS	533	AY714161
29	Meliphagidae	*Myzomela*	*obscura*	Dusky honeyeater	AUS	533	AY714162
30	Meliphagidae	*Philemon*	*buceroides*	Helmeted friarbird	PNG	533	AY714163
31	Meliphagidae	*Myzomela*	*nigrita*	Papuan black myzomela	PNG	533	AY714164
31	Pachycephalidae	*Colluricincla*	*megarhyncha*	Little shrike-thrush	PNG	533	AY714164
32	Meliphagidae	*Melilestes*	*megarhynchus*	Long-billed honeyeater	PNG	533	AY714165
32	Pomatostomidae	*Pomatostomus*	*isodorei*	Rufous babbler	PNG	533	AY714165
33	Meliphagidae	*Acanthorhynchus*	*tenuirostris*	Eastern spinebill	AUS	533	AY714166
34	Petroicidae	*Tregellasia*	*capito*	Pale-yellow robin	AUS	533	AY714167
35	Petroicidae	*Tregellasia*	*capito*	Pale-yellow robin	AUS	533	AY714168
36	Petroicidae	*Tregellasia*	*capito*	Pale-yellow robin	AUS	533	AY714169
37	Petroicidae	*Tregellasia*	*capito*	Pale-yellow robin	AUS	533	AY714170
38	Petroicidae	*Heteromyias*	*albispecularis*	Grey-headed robin	AUS	533	AY714171
39	Petroicidae	*Heteromyias*	*albispecularis*	Grey-headed robin	AUS	533	AY714172
40	Petroicidae	*Tregellasia*	*capito*	Pale-yellow robin	AUS	533	AY714173
41	Petroicidae	*Eopsaltria*	*australis*	Eastern yellow robin	AUS	533	AY714174
42	Petroicidae	*Eopsaltria*	*australis*	Eastern yellow robin	AUS	533	AY714175
43	Melanocharitidae	*Melanocharis*	*nigra*	Black berrypecker	PNG	533	AY714176
44	Meliphagidae	*Meliphaga*	*lewinii*	Lewin's honeyeater	AUS	533	AY714177

Appendix I *Continued*

Lineage	Host information Family	Genus	Species	Common name	Locality	bp	GenBank Acc. No.
45	Meliphagidae	*Lichenostomus*	*frenatus*	Bridled honeyeater	AUS	533	AY714178
46	Meliphagidae	*Lichenostomus*	*frenatus*	Bridled honeyeater	AUS	533	AY714179
47	Orthonychidae	*Orthonyx*	*spaldingii*	Chowchilla	AUS	533	AY714180
48	Meliphagidae	*Xanthotis*	*macleayana*	Macleay's honeyeater	AUS	533	AY714181
49	Meliphagidae	*Xanthotis*	*flaviventer*	Tawny-breasted honeyeater	PNG	533	AY714182
50	Meliphagidae	*Xanthotis*	*flaviventer*	Tawny-breasted honeyeater	PNG	533	AY714183
51	Meliphagidae	*Melipotes*	*fumigatus*	Smoky honeyeater	PNG	533	AY714184
52	Monarchidae	*Monarcha*	*guttula*	Spot-winged monarch	PNG	533	AY714185
52	Monarchidae	*Arses*	*telescophthalmus*	Frilled monarch	PNG	533	AY714185
53	Pachycephalidae	*Pachycephala*	*simplex*	Grey whistler	PNG	533	AY714186
54	Pachycephalidae	*Pitohui*	*kirhocephalus*	Variable pitohui	PNG	533	AY714187
55	Pachycephalidae	*Pitohui*	*kirhocephalus*	Variable pitohui	PNG	533	AY714188
55	Meliphagidae	*Lichenostomus*	*hindwoodi*	Eungella honeyeater	AUS	533	AY714188
56	Pachycephalidae	*Pitohui*	*dichrous*	Hooded pitohui	PNG	533	AY714189
57	Paradisaeidae	*Ptilloris*	*magnificus*	Magnificent riflebird	PNG	533	AY714190
58	Monarchidae	*Monarcha*	*chrysomela*	Golden monarch	PNG	533	AY714191
59	Cracticidae	*Cracticus*	*quoyi*	Black butcherbird	PNG	533	AY714192
60	Columbidae	*Macropygia*	*amboinensis*	Brown cuckoo-dove	PNG	533	AY714193
61	Nectariniidae	*Nectarinia*	*aspasia*	Black sunbird	PNG	295	AY714194
62	Pachycephalidae	*Pachycephala*	*pectoralis*	Golden whistler	AUS	533	AY714195
62	Monarchidae	*Rhipidura*	*fuliginosa*	Grey fantail	AUS	533	AY714195
63	Monarchidae	*Rhipidura*	*rufifrons*	Rufous fantail	AUS	533	AY714196
64	Meliphagidae	*Meliphaga*	*notata*	Yellow-spotted honeyeater	AUS	533	AY714197
65	Meliphagidae	*Meliphaga*	*analoga*	Mimic honeyeater	PNG	533	AY714198
65	Meliphagidae	*Meliphaga*	*lewinii*	Lewin's honeyeater	AUS	533	AY714198
66	Monarchidae	*Monarcha*	*trivirgatus*	Spectacled monarch	AUS	533	AY714199
66	Monarchidae	*Monarcha*	*kaupi*	Pied monarch	AUS	533	AY714199
66	Petroicidae	*Heteromyias*	*albispecularis*	Grey-headed robin	AUS	533	AY714199
67	Monarchidae	*Myiagra*	*alecto*	Shining flycatcher	PNG	533	AY714200
68	Pachycephalidae	*Eulecestoma*	*nigripectus*	Wattled ploughbill	PNG	533	AY714201
69	Pittidae	*Pitta*	*versicolour*	Noisy pitta	AUS	533	AY714202
70	Acanthizidae	*Acanthiza*	*katherina*	Mountain thornbill	AUS	533	AY714203
70	Pachycephalidae	*Colluricincla*	*megarhyncha*	Little shrike-thrush	AUS	533	AY714203
70	Alcedinidae	*Tanysiptera*	*galatea*	Common paradize kingfisher	PNG	533	AY714203
71	Pachycephalidae	*Pitohui*	*incertus*	White-bellied pitohui	PNG	533	AY714204
72	Ptilonorhynchidae	*Scenopoeetes*	*dentirostris*	Tooth-billed bowerbird	AUS	533	AY714205
72	Ptilonorhynchidae	*Ailuroedes*	*melanotis*	Spotted catbird	AUS	533	AY714205
72	Meliphagidae	*Lichenostomus*	*frenatus*	Bridled honeyeater	AUS	533	AY714205
72	Acanthizidae	*Sericornis*	*magnirostris*	Large-billed scrubwren	AUS	533	AY714205
72	Pachycephalidae	*Colluricincla*	*boweri*	Bower's shrike-thrush	AUS	533	AY714205
72	Monarchidae	*Monarcha*	*trivirgatus*	Spectacled monarch	AUS	533	AY714205
73	Pachycephalidae	*Pitohui*	*dichrous*	Hooded pitohui	PNG	533	AY714206
73	Acanthizidae	*Sericornis*	*nouhuysi*	Large scrubwren	PNG	469	AY714206
74	Meliphagidae	*Meliphaga*	*notata*	Yellow-spotted honeyeater	AUS	533	AY714207
75	Ptilonorhynchidae	*Ailuroedes*	*melanotis*	Spotted catbird	AUS	485	AY714208
76	Columbidae	*Chalcophaps*	*indica*	Emerald dove	PNG	295	AY714209
77	Acanthizidae	*Crateroscelis*	*robusta*	Mountain mouse-warbler	PNG	295	AY714210
78	Acanthizidae	*Crateroscelis*	*murina*	Rusty mouse-warbler	PNG	295	AY714211

AUS, Australia; PNG, Papua New Guinea.

Appendix II

Frequency of detection of *Haemoproteus* (H), *Plasmodium* (P), unknown genus (U) or mixed infection (M) across host families from Australia (AUS) and Papua New Guinea (PNG). Composition of mixed infections is indicated at right

Host	Location	Total	H	P	U	M	INF	Mixed
Accipitridae								
Accipiter poliocephalus	PNG	1					0	
Megapodidae								
Megapodius reinwardt	PNG	1					0	
Columbidae								
Chalcophaps indica	PNG	3	1	1		1	3	H
Chalcophaps stephani	PNG	1	1				1	
Ducula pinon	PNG	1					0	
Macropygia amboinensis	PNG	3	1				1	
Ptilinopus magnificus	PNG	2	1				1	
Ptilinopus pulchellus	PNG	1					0	
Ptilinopus rivoli	PNG	2	1				1	
Ptilinopus superbus	PNG	4	2		1	1	4	H
Podargidae								
Podargus ocellatus	PNG	2			1		1	
Aegothelidae								
Aegotheles bennettii	PNG	2					0	
Alcedinidae								
Alcedo azurea	PNG	1					0	
Alcedo pusilla	PNG	1					0	
Halcyon chloris	PNG	1					0	
Halcyon sancta	PNG	1					0	
Halcyon torotoro	PNG	4	2		1		3	
Melidora macrorhina	PNG	1					0	
Tanysiptera danae	PNG	4					0	
Tanysiptera galatea	PNG	10	3	1			4	
Pittidae								
Pitta versicolour	AUS	3		1			1	
Climacteridae								
Cormobates leucophaeus	AUS	3					0	
Ptilonorhynchidae								
Ailuroedus melanotis	AUS	8	3	2		1	6	P
Scenopoeetes dentirostris	AUS	7	2	2		2	6	P, PH
Acanthizidae								
Acanthiza katherina	AUS	8		1			1	
Crateroscelis murina	PNG	5	1	3			4	
Crateroscelis robusta	PNG	6		3			3	
Gerygone mouki	AUS	2					0	
Oreoscopus gutturalis	AUS	6			1		1	
Sericornis citreogularis	AUS	12	2				2	
Sericornis frontalis	AUS	7					0	
Sericornis keri	AUS	4					0	
Sericornis magnirostris	AUS	14	5			1	6	PH
Sericornis nouhuysi	PNG	1		1			1	
Sericornis papuensis	PNG	2					0	
Sericornis perspicillatus	PNG	2					0	
Meliphagidae								
Acanthorhynchus tenuirostris	AUS	7	1		1		2	
Lichenostomus frenatus	AUS	8	6	1			7	
Lichenostomus hindwoodi	AUS	4	1				1	
Melilestes megarhynchus	PNG	3	1				1	
Meliphaga analoga	PNG	1		1			1	
Meliphaga aruensis	PNG	4		1			1	
Meliphaga lewinii	AUS	16	5	2			7	
Meliphaga notata	AUS	3		2			2	

Appendix II *Continued*

Host	Location	Total	H	P	U	M	INF	Mixed
Melipotes fumigatus	PNG	5	2				2	
Myzomela nigrita	PNG	2	1			1	2	H
Myzomela obscura	AUS	3	2			1	3	H
Oedistoma iliolophus	PNG	6	6				6	
Philemon buceroides	PNG	1	1				1	
Xanthotis flaviventer	PNG	2	2				2	
Xanthotis macleayana	AUS	5	4				4	
Petroicidae								
Amalocichla incerta	PNG	2					0	
Eopsaltria australis	AUS	3	2		1		3	
Heteromyias albispecularis	AUS	17	5	1		5	11	H
Melanodryas cucullata	PNG	1					0	
Petroica rosea	PNG	1					0	
Tregellasia capito	AUS	10	3			4	7	H
Orthonychidae								
Orthonyx spaldingii	AUS	4	1				1	
Pomatostomidae								
Pomatostomus isidorei	PNG	5	1				1	
Cinclosomatidae								
Cinclosoma ajax	PNG	2					0	
Psophodes olivaceus	AUS	7					0	
Psophodes olivaceus	PNG	1					0	
Pachycephalidae								
Colluricincla boweri	AUS	8	1	1			2	
Colluricincla harmonica	PNG	1					0	
Colluricincla megarhyncha	AUS	11	4			1	5	PH
Colluricincla megarhyncha	PNG	16	4	1			5	
Colluricincla woodwardi	PNG	1				1	1	?
Eulecestoma nigripectus	PNG	1		1			1	
Falcunculus frontatus	PNG	1			1		1	
Pachycephala melanura	PNG	2					0	
Pachycephala olivacea	PNG	1			1		1	
Pachycephala pectoralis	AUS	11		1			1	
Pachycephala pectoralis	PNG	1					0	
Pachycephala schlegelii	PNG	1					0	
Pachycephala simplex	PNG	3	1		1		2	
Pitohui cristatus	PNG	3	2				2	
Pitohui dichrous	PNG	10	2	1			3	
Pitohui ferrugineus	PNG	14	1		1	1	3	?
Pitohui incertus	PNG	3	1	1			2	
Pitohui kirhocephalus	PNG	5	1		1	2	4	PH
Rhagologus leucostigma	PNG	1					0	
Paradisaeidae								
Cicinnurus magnificus	PNG	1					0	
Paradiseae raggiana	PNG	1			1		1	
Ptilloris magnificus	PNG	1	1				1	
Cracticidae								
Cracticus quoyi	PNG	2	2				2	
Campephagidae								
Lalage leucomela	PNG	1	1				1	
Dicruridae								
Chaetorynchus papuensis	PNG	1					0	
Dicrurus hottentottus	PNG	2					0	
Monarchidae								
Arses telescopthalmus	PNG	3	1			1	2	H
Machaerirynchus flaviventer	AUS	3	1	1			2	
Monarcha chrysomela	PNG	3	3				3	

Appendix II *Continued*

Host	Location	Total	H	P	U	M	INF	Mixed
Monarcha guttula	PNG	5	3				3	
Monarcha kaupi	AUS	3		3			3	
Monarcha trivirgatus	AUS	12		3		1	4	P
Myiagra alecto	PNG	3	1	2			3	
Rhipidura albolimbata	PNG	2					0	
Rhipidura atra	PNG	3					0	
Rhipidura brachyrhyncha	PNG	1					0	
Rhipidura fulginosa	AUS	7		2			2	
Rhipidura rufifrons	PNG	2					0	
Rhipidura rufifrons	AUS	1		1			1	
Rhipidura rufiventris	PNG	6	3			1	5	H
Sylviidae								
Phylloscopus trivirgatus	PNG	1					0	
Muscicapidae								
Zoothera lunulata	AUS	2	1				1	
Melanocharitidae								
Melanocharis nigra	PNG	1	1				1	
Nectariniidae								
Nectarinia aspasia	PNG	2				1	1	PH
Zosteropidae								
Zosterops fuscicapillus	PNG	3	1			1	2	H
Zosterops griseotinctus	PNG	2					0	
Passeridae								
Erythrura trichroa	PNG	1					0	

AUS, Australia; PNG, Papua New Guinea.