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Molecular characterization of *Babesia peircei* and *Babesia ugwidiensis* provides insight into the evolution and host specificity of avian piroplasmids



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ABSTRACT

There are 16 recognized species of avian-infecting *Babesia* spp. (Piroplasmida: Babesiidae). While the classification of piroplasmids has been historically based on morphological differences, geographic isolation and presumed host and/or vector specificities, recent studies employing gene sequence analysis have provided insight into their phylogenetic relationships and host distribution and specificity. In this study, we analyzed the sequences of the 18S rRNA gene and ITS-1 and ITS-2 regions of two *Babesia* species from South African seabirds: *Babesia peircei* from African penguins (*Spheniscus demersus*) and *Babesia ugwidiensis* from Bank and Cape cormorants (*Phalacrocorax neglectus* and *P. capensis*, respectively). Our results show that avian *Babesia* spp. are not monophyletic, with at least three distinct phylogenetic groups. *B. peircei* and *B. ugwidiensis* are closely related, and fall within the same phylogenetic group as *B. ardeae* (from herons *Ardea cinerea*), *B. poelea* (from boobies *Sula* spp.) and *B. uriae* (from murrelets *Uria aalge*). The validity of *B. peircei* and *B. ugwidiensis* as separate species is corroborated by both morphological and genetic evidence. On the other hand, our results indicate that *B. poelea* might be a synonym of *B. peircei*, which in turn would be a host generalist that infects seabirds from multiple orders. Further studies combining morphological and molecular methods are warranted to clarify the taxonomy, phylogeny and host distribution of avian piroplasmids.

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1. Introduction

There are currently 16 recognized species of avian-infecting *Babesia* spp. (Piroplasmida: Babesiidae) (Peirce, 2000, 2005; Yabsley et al., 2009; Peirce and Parsons, 2012). In addition, several uncharacterized or unnamed *Babesia* spp. have been reported (Peirce, 2000; Beaufrère et al., 2007; Savage et al., 2009;

Paparini et al., 2014; Martínez et al., 2015; Vanstreels et al., 2015; Montero et al., 2016). Historically, the classification of piroplasmids has been primarily based on morphological differences, presumed host and/or vector specificities, or geographic isolation (Peirce, 2000). In some cases, *Babesia* spp. sharing similar morphology were described as separate species based on the presumption that these parasites are host-specific at the family or order level (Peirce, 2005). For instance, Work and Rameyer (1997) in their description of *B. poelea* pointed out that based on morphology alone, the parasite would be classified as *B. moshkovskii* based on recommendations of Laird and Lari (1957) and Levine (1971); however, they believed that because the parasite infected a pelagic species isolated from previous terrestrial hosts of

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Babesia and lack of presumed tick vectors associated with other avian *Babesia*, it was a unique species. Similarly, morphological similarity has been noted among *B. poeala*, *B. peircei* and *B. uriae*, but the fact that they were found in different avian orders has led to the interpretation that they are distinct parasite species (Peirce, 2000; Yabsley et al., 2006, 2009).

In recent years, studies employing gene sequence analysis have increased the knowledge of piroplasmid diversity, their phylogenetic relationships and host distribution (Schnittger et al., 2012). In some cases, this has led to complications due to recognition of parasites that have significant morphological similarities but have sufficient genetic variation to warrant separate species designations (i.e., cryptic species) (Birkenheuer et al., 2008; Holman et al., 2009; Mandal et al., 2014). In other cases, the phylogenetic data has raised doubt on the presumption of family-level host specificity for some of these parasites (Paparini et al., 2014; Vanstreels et al., 2015).

Sequences of the 18S rRNA gene are available for five morpho-species of avian piroplasmids: *B. bennetti* from yellow-legged gulls (*Larus cachinnans*) (Criado et al., 2006), *B. poeala* from masked and brown boobies (*Sula dactylatra* and *S. leucogaster*, respectively) (Yabsley et al., 2006; Quillfeldt et al., 2014), *B. kiwiensis* from North Island brown kiwis (*Apteryx mantelli*) (Jefferies et al., 2008), *B. uriae* from common murre (Uria aalge) (Yabsley et al., 2009), and *B. ardeae* from grey herons (*Ardea cinerea*) (Chavatte et al., 2017). Phylogenetic analysis of these sequences reveals three separate clusters, with *B. ardeae*, *B. poeala* and *B. uriae* being grouped together (Chavatte et al., 2017).

In this study, we evaluate the phylogenetic relationships of another two morphospecies, *B. peircei* from African penguins (*Spheniscus demersus*) and *B. ugwidensis* from Cape and Bank cormorants (*Phalacrocorax capensis* and *P. neglectus*, respectively), and discuss the implications of our results with regards to the evolution and host specificity of avian piroplasmids.

2. Materials and methods

Blood was collected from Cape and Bank cormorants and African penguins admitted for rehabilitation at the Southern African Foundation for the Conservation of Coastal Birds (SANCCOB) facility in Cape Town, Western Cape, South Africa (33°50′02″S 18°29′29″E) and African penguins at the Penguins Eastern Cape (PEC) facility in Cape St. Francis, Eastern Cape, South Africa (34°12′44″S 24°50′08″E). Blood smears were freshly prepared, fixed with methanol, stained with a modified Wright–Giemsa stain (Kyro-Quick stain, Kyron Laboratories, Benrose, South Africa), and examined under light microscopy. Penguin parasites were identified as *Babesia peircei* based on the distal location of the nucleus within merozoites, the presence of amoeboid tetrads (“cow’s udder” form), and the general consistency with the morphological characteristics as described by Earlé et al. (1993). Cormorant parasites were identified as *Babesia ugwidensis* based on the proximal location of the nucleus within merozoites, the rarity of “Maltese cross” schizont forms, and the general consistency with the morphological characteristics as described by Peirce and Parsons (2012).

DNA was extracted from ~10 µL of ethanol preserved whole blood or from a dried fixed blood spot using the GFX Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, New Jersey) following the manufacturer’s protocol. Primary outside amplification for the *Babesia* sp. 18S rRNA gene was conducted as described in Yabsley et al. (2006, 2009). Briefly, 5 µL of DNA was added to 20 µL of a master mix containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each dNTP (Promega, Madison, Wisconsin), 2.5 units Taq DNA Polymerase (Promega,

and 0.8 µM of primers 5.1 and B. Cycling parameters were: 94 °C for 1 min followed by 30 cycles of 94 °C for 1 min, 48 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 5 min. For the nested PCR, 1 µL of primary product was used as template in a 25 µL reaction containing the same PCR components except primers, 5.1V2 and 3.1 or F and R were used. Cycling conditions were the same as primary reaction except the annealing temperature was 52 °C or 50 °C, respectively. The internal transcribed spacer (ITS) regions 1 and 2 were amplified as previously described using primers ITS-15C and ITS-13B in a primary reaction and the primers ITS-15D and ITS-13C in the secondary reaction for ITS-1 and primers FOR7 and REV7 in the secondary reaction for ITS-2 (Shock et al., 2012). Bi-directional sequencing of amplification products was conducted using the same primers; resulting sequences were deposited in GenBank (accession codes MF288008–MF288031). DNA extraction, primary amplification, secondary amplification, and product analysis were performed in separate dedicated laboratory areas, and negative water controls were included in each set of DNA extractions and PCR reactions.

Phylogenetic relationships of the *Babesia* spp. in this study were inferred using published sequences of mammalian-infecting *Babesia*, in addition to avian-infecting *Babesia* spp. from published studies (Criado et al., 2006; Yabsley et al., 2006, 2009; Jefferies et al., 2008; Paparini et al., 2014; Quillfeldt et al., 2014; Martínez et al., 2015; Montero et al., 2016; Chavatte et al., 2017). *Cardiosporidium cionae* was included as an out-group (Schnittger et al., 2012). Sequences were aligned using ClustalW (Thompson et al., 1997) as implemented in MEGA 6.06 (Tamura et al., 2011). Phylogenetic analyses used a trimmed alignment of 1450 bp for the 18S rRNA gene. To include the shorter 18S rRNA gene sequence of *Babesia* sp. obtained by Montero et al. (2016) in the analysis, another alignment trimmed to 303 bp was used. Unfortunately, there are not enough publicly-available sequences for a comprehensive comparison for the ITS-1 and ITS-2 regions; as a result, analyses were limited to comparing the sequences obtained in this study to those of *B. poeala* (Yabsley et al., 2006) and *B. uriae* (Yabsley et al., 2009). Maximum likelihood phylogenetic trees were produced using MEGA 6.06, with 5000 bootstrap replications. Models of nucleotide evolution for the 18S rRNA gene (GTR + I + Γ) and for the ITS-1 (GTR + Γ) and ITS-2 regions (HKY + I) were selected using jModelTest 2.1.10 (Darrriba et al., 2012). Pairwise estimates of evolutionary divergence were produced with MEGA 6.06 using a maximum composite likelihood model, with a gamma distribution (shape parameter = 1), including transitions and transversions, and excluding ambiguous positions for each sequence pair.

3. Results

Full-length 18S rRNA gene sequences were obtained for three samples with *B. ugwidensis* (two Cape cormorants, one Bank cormorant) and two samples with *B. peircei*; another two partial sequences of *B. ugwidensis* were also obtained from Cape cormorants. All *B. ugwidensis* 18S rRNA gene sequences were identical to one another; the same occurred for the two *B. peircei* sequences. Phylogenetic analysis of the 18S rRNA gene sequences (Fig. 1, Supplementary Data S1) revealed that avian *Babesia* are organized in three groups, with *B. ugwidensis* and *B. peircei* falling in the same group as *B. ardeae*, *B. poeala*, *B. uriae*, and unidentified strains of *Babesia* from little penguins (*Eudyptula minor*) from Australia, and Australasian gannets (*Morus serrator*), red-billed gulls (*Chroicocephalus scopulinus*) and white-fronted terns (*Sterna striata*) from New Zealand. Pairwise estimates of evolutionary divergence between the 18S rRNA gene sequences obtained in this study and those of other avian-infecting *Babesia* are provided in Table 1.

ITS-1 region sequences were obtained from seven samples with

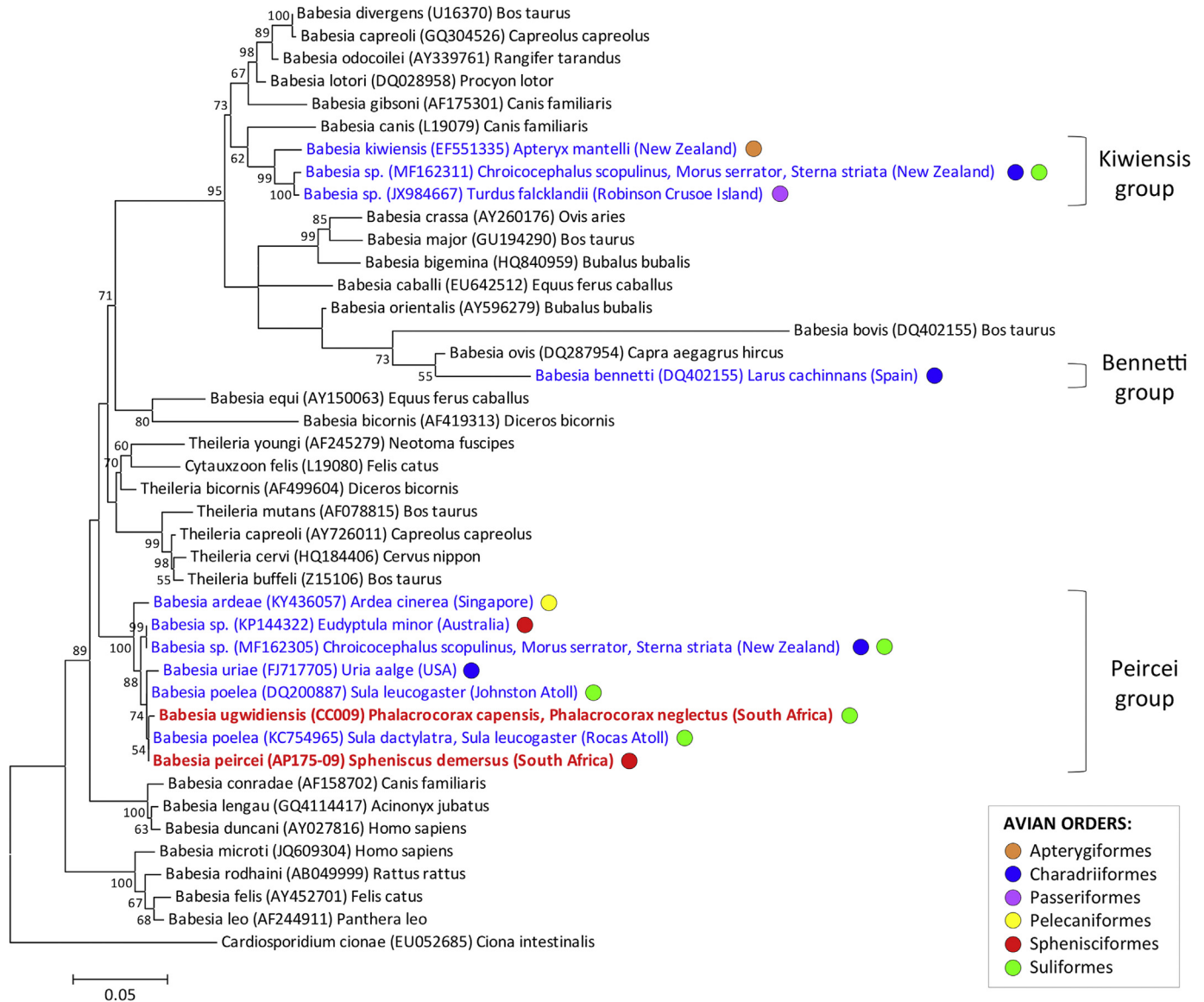


Fig. 1. Maximum likelihood phylogenetic tree of the 18S rRNA gene sequences (1450 bp) of the studied *Babesia* lineages. Sequences obtained in this study are emphasized in red, and those of other avian-infecting lineages are shown in blue. For each sequence, the following information is provided: morphospecies (individual identification or GenBank code) host species. For avian-infecting lineages, the host order is indicated with colored circles (see legend). Branch lengths are drawn proportionally to evolutionary distance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

B. ugwidiensis (six Cape cormorants, one Bank cormorant) and four samples with *B. peircei*. For *B. ugwidiensis*, only two sequences were identical, and overall 97.2% of sites were conserved (447/460). For *B. peircei*, three sequences were identical but differed significantly from the fourth sequence, with 95.4% of conserved sites (420/440); most of these differences were related to the deletion of a 15 bp segment. ITS-2 region sequences were obtained from four samples with *B. ugwidiensis* (three Cape cormorants, one Bank cormorant) and two samples with *B. peircei*. For *B. ugwidiensis*, all sequences from Cape cormorants were identical, and the Bank cormorant sequence only differed in one site (99.6% conserved sites; 273/274). For *B. peircei*, three separate nucleotide substitutions were noted (98.9% conserved sites; 271/274). Phylogenetic analysis of the ITS-1 and ITS-2 regions revealed that the lineages clustered according to their host family (Fig. 2, Tables 2 and 3). For both ITS regions, pairwise estimates of evolutionary divergence within *B. ugwidiensis* (ITS-1: 0 to 1.58 base substitutions per 100 sites; ITS-2: 0 to 0.37)

and within *B. peircei* (ITS-1: 0 to 0.74; ITS-2: 1.09) were considerably lower than the divergence between *B. ugwidiensis* and *B. peircei* (ITS-1: 8.77 to 11.11; ITS-2: 15.05 to 17.71).

Interestingly, the 18S rRNA gene evolutionary divergence between *B. peircei* and *B. poelea* (0.07–0.15 base substitutions per 100 sites) was similar to the divergence between *B. poelea* from different locations (0.07); in contrast, the divergence between *B. peircei* and *Babesia* sp. from little penguins sampled in Australia was considerably higher (1.06). Additionally, the evolutionary divergence between *B. peircei* and *B. poelea* was remarkably low for both ITS-1 (2.68–2.81) and ITS-2 regions (2.21–3.42).

4. Discussion

4.1. Evolution of avian piroplasmids

Most known avian-infecting *Babesia* spp. have yet to undergo

Table 1
Estimates of evolutionary divergence (base substitutions per 100 sites) of 18S rRNA gene sequences (1450 bp, unless noted otherwise) of avian *Babesia* spp.

Sequence	2	3	4	5	6	7	8	9	10	11	12	13*
1. <i>B. ugwidiensis</i> (CC009) <i>P. capensis</i> , <i>P. neglectus</i> (South Africa)	0.22	0.30	0.37	0.90	2.46	12.56	10.62	1.29	1.37	12.47	12.69	8.60
2. <i>B. peircei</i> (AP175-09) <i>S. demersus</i> (South Africa)		0.07	0.15	0.75	2.22	12.16	10.53	1.06	1.14	11.97	12.30	8.20
3. <i>B. poeala</i> (KC754965) <i>S. dactylatra</i> , <i>S. leucogaster</i> (Rocas Atoll)			0.07	0.83	2.14	12.16	10.61	0.98	1.06	11.96	12.29	8.17
4. <i>B. poeala</i> (DQ200887) <i>S. leucogaster</i> (Johnston Atoll)				0.75	2.22	12.05	10.62	0.90	0.98	11.85	12.18	7.74
5. <i>B. uriae</i> (FJ717705) <i>U. aalge</i> (USA)					2.69	12.40	10.73	1.44	1.52	12.07	12.19	8.60
6. <i>B. ardeae</i> (KY436057) <i>A. cinerea</i> (Singapore)						12.14	11.01	2.37	2.38	12.13	12.25	12.48
7. <i>B. kiwiensis</i> (EF551335) <i>A. mantelli</i> (NZ)							10.25	12.16	12.17	3.57	3.48	22.38
8. <i>B. bennetti</i> (DQ402155) <i>L. cachinnans</i> (Spain)								10.62	10.64	9.76	9.66	19.22
9. <i>B. sp.</i> (KP144322) <i>E. minor</i> (Australia)									0.07	11.65	11.87	4.29
10. <i>B. sp.</i> (MF162305) <i>C. scopulinus</i> , <i>M. serrator</i> , <i>S. striata</i> (NZ)										11.66	11.88	4.31
11. <i>B. sp.</i> (MF162311) <i>C. scopulinus</i> , <i>M. serrator</i> , <i>S. striata</i> (NZ)											0.70	23.47
12. <i>B. sp.</i> (JX984667) <i>T. falcklandii</i> (Robinson Crusoe Island)												22.67
13. <i>B. sp.</i> (KT800053) <i>P. antarcticus</i> (South Shetland Islands)*												

*Estimates based on a partial sequence (303 bp).

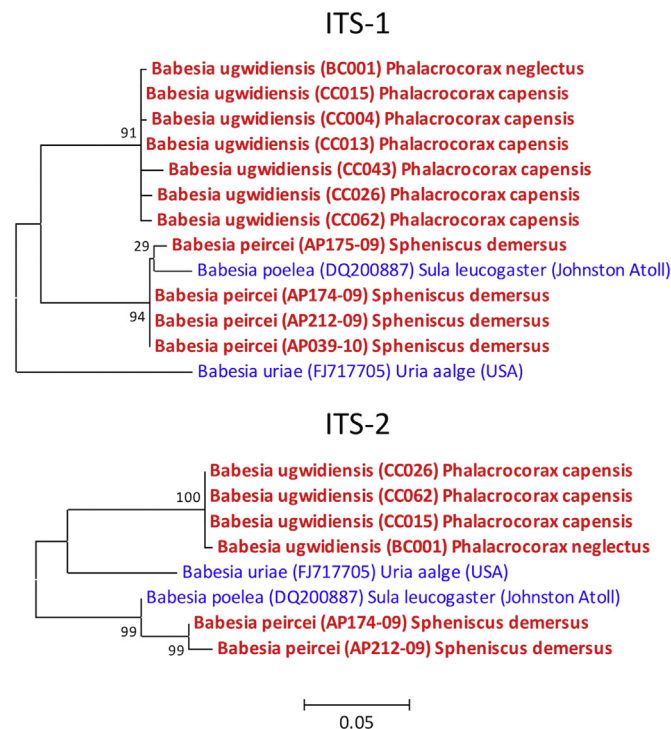


Fig. 2. Maximum likelihood phylogenetic tree of the ITS-1 (445 bp) and ITS-2 regions sequences (290 bp) of select avian-infecting *Babesia* lineages. Sequences identified in this study are emphasized in red, and those of other avian-infecting lineages are shown in blue. For each sequence, the following information is provided: morphospecies (individual identification or Genbank code) host species. Branch lengths are drawn proportionally to evolutionary distance (scale bar shown corresponds to both trees). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecular characterization, and as a result our understanding of their evolution and phylogeography is limited by the few species that have been the subject of such studies, most of which focused on aquatic birds. Our results reveal that the avian-infecting *Babesia* spp. genetically characterized thus far are not monophyletic, but instead correspond to three paraphyletic groups that emerge from mammalian-infecting *Babesia* spp. (Fig. 1). The ‘Kiwiensis group’ comprises *B. kiwiensis* and other unidentified *Babesia* sp., and has been recorded in both terrestrial (Apterygiformes, Passeriformes) and aquatic birds (Charadriiformes, Suliformes) (Fig. 3). The ‘Bennetti group’ thus far only contains one species, *B. bennetti*, which was described from aquatic birds (Charadriiformes). The large

‘Peircei group’ corresponds to *B. ardeae*, *B. poeala*, *B. peircei*, *B. ugwidiensis* and *B. uriae* and other unidentified *Babesia* spp., all of which are from aquatic birds (Charadriiformes, Pelecaniformes, Sphenisciformes, Suliformes). From a geographic perspective, the Peircei group is broadly distributed worldwide, whereas the Kiwiensis group has only been recorded in the Pacific Ocean and the Bennett group is only known from the Mediterranean Sea (Fig. 4).

When the broader evolutionary history of piroplasmids is considered, the Kiwiensis group is part of Clade VI (*sensu* Schnittger et al., 2012), a strongly supported monophyletic group that comprises *Babesia* spp. from carnivores, rodents and ungulates which is also referred to as ‘*Babesia sensu stricto*’. The Peircei group is a sister to all other clades except Clades I and II, constituting a moderately supported group that also includes *Babesia* sp. isolates from ungulates, carnivores and accidental infections of humans, and which is sometimes referred to as the “Western clade” due to the fact that when first described, samples originated from the Western states of the USA. But this group now includes representatives from various African mammals as well (e.g., Bosman et al., 2010; McDermid et al., 2017). The position of the Bennett group is not clear, and it could either be part of clade VI or represent a sister clade that has yet to be resolved.

The polyphyletic origin (i.e. derived from more than one ancestor) of avian piroplasmids is not surprising considering what is known about their mammalian counterparts, wherein well studied hosts (e.g. humans and domestic animals) are infected by a number of piroplasmid species with polyphyletic origins (Schnittger et al., 2012). In this context, the emergence of avian piroplasmids amidst their mammalian counterparts testifies to host switching of ticks between birds and mammals; this is consistent with the occasional records of tick transmission across these vertebrate classes (Ogrzewalska et al., 2011; Muñoz-Leal et al., 2013; Muñoz-Leal and Gonzalez-Acuña, 2015).

In a separate analysis (Supplementary Data S1), the sequence of *Babesia* sp. from chinstrap penguins (*Pygoscelis antarcticus*) (Montero et al., 2016) was not narrowly clustered with any other published sequences of avian- or mammalian-infecting *Babesia*, and could potentially represent a separate phylogenetic group within the ‘*Babesia sensu stricto*’ clade (Clade VI in Schnittger et al., 2012). However, because the 18S rRNA gene sequence currently available for this parasite is short (274 bp), further molecular studies are needed before its phylogenetic relationships can be confidently evaluated. Additionally, it should be noted that there are numerous *Babesia* spp. infecting different avian orders that have yet to undergo molecular characterization (Fig. 3) (Peirce, 2000), and future studies might reveal additional evolutionary

Table 2Estimates of evolutionary divergence (base substitutions per 100 sites) of ITS-1 region sequences (445 bp) of avian *Babesia* spp.

Sequence	2	3	4	5	6	7	8	9	10	11	12	13
1. <i>B. ugwidiensis</i> (BC001) <i>P. neglectus</i> (South Africa)	0.25	0.51	0.25	0.77	1.30	0.76	9.15	9.15	9.15	9.90	9.65	12.04
2. <i>B. ugwidiensis</i> (CC015) <i>P. capensis</i> (South Africa)		0.25	0.00	0.51	1.03	0.50	8.77	8.77	8.77	9.51	9.26	11.62
3. <i>B. ugwidiensis</i> (CC004) <i>P. capensis</i> (South Africa)			0.25	0.77	1.30	0.76	9.15	9.15	9.15	9.90	9.65	12.04
4. <i>B. ugwidiensis</i> (CC013) <i>P. capensis</i> (South Africa)				0.51	1.03	0.50	8.77	8.77	8.77	9.51	9.26	11.62
5. <i>B. ugwidiensis</i> (CC026) <i>P. capensis</i> (South Africa)					1.58	1.03	9.53	9.53	9.53	10.30	10.04	12.46
6. <i>B. ugwidiensis</i> (CC043) <i>P. capensis</i> (South Africa)						1.57	10.31	10.31	10.31	11.11	10.85	12.46
7. <i>B. ugwidiensis</i> (CC062) <i>P. capensis</i> (South Africa)							9.46	9.46	9.46	10.22	9.97	11.54
8. <i>B. peircei</i> (AP039-10) <i>S. demersus</i> (South Africa)								0.00	0.00	0.74	2.68	14.37
9. <i>B. peircei</i> (AP174-09) <i>S. demersus</i> (South Africa)									0.00	0.74	2.68	14.37
10. <i>B. peircei</i> (AP212-09) <i>S. demersus</i> (South Africa)										0.74	2.68	14.37
11. <i>B. peircei</i> (AP175-09) <i>S. demersus</i> (South Africa)											2.81	14.96
12. <i>B. poeala</i> (DQ200887) <i>S. leucogaster</i> (Johnston Atoll)												13.75
13. <i>B. uriae</i> (FJ717705) <i>U. aalge</i> (USA)												

Table 3Estimates of evolutionary divergence (base substitutions per 100 sites) of ITS-2 region sequences (290 bp) of avian *Babesia* spp.

Sequence	2	3	4	5	6	7	8
1. <i>B. ugwidiensis</i> (BC001) <i>P. neglectus</i> (South Africa)	0.37	0.37	0.37	15.69	17.71	12.52	11.55
2. <i>B. ugwidiensis</i> (CC015) <i>P. capensis</i> (South Africa)		0.00	0.00	15.05	17.02	11.93	11.00
3. <i>B. ugwidiensis</i> (CC026) <i>P. capensis</i> (South Africa)			0.00	15.05	17.02	11.93	11.00
4. <i>B. ugwidiensis</i> (CC062) <i>P. capensis</i> (South Africa)				15.05	17.02	11.93	11.00
5. <i>B. peircei</i> (AP174-09) <i>S. demersus</i> (South Africa)					1.09	2.21	15.02
6. <i>B. peircei</i> (AP212-09) <i>S. demersus</i> (South Africa)						3.42	16.93
7. <i>B. poeala</i> (DQ200887) <i>S. leucogaster</i> (Johnston Atoll)							13.06
8. <i>B. uriae</i> (FJ717705) <i>U. aalge</i> (USA)							

branches of avian piroplasmids.

4.2. Host specificity and species differentiation within the *peircei* group

The hosts of the Peircei group have several life history characteristics in common (i.e., all are aquatic birds), are relatively closely related (Fig. 3), and are known to share tick species (Dietrich et al., 2011; Muñoz-Leal and González-Acuña, 2015). Several authors have noted that there are morphological similarities among the species of the Peircei group, particularly in relation to the presence of amoeboid tetrads and the distal positioning of the chromatin within merozoites (except in *B. ardeae*; Chavatte et al., 2017); in fact, the case has been made that it would be difficult (if not impossible) to distinguish *B. peircei*, *B. poeala* and *B. uriae* solely on the basis of morphology (Peirce, 2000; Yabsley et al., 2009; Vanstreels et al., 2015). The recent emergence of genetic evidence showing that these parasites are closely related raises further questions with regards to whether these taxa represent a species complex that comprises multiple species with narrow host-specificity, or whether some of these taxa might actually be synonyms and represent parasites that are shared by multiple families/orders of aquatic birds.

Our 18S rRNA gene phylogenetic analysis of *B. peircei* and *B. ugwidiensis* shows that these parasites are very closely related, with as little as 0.22 expected base substitutions per 100 sites. However, although the 18S rRNA gene has been widely used in most evolutionary studies of piroplasmids, this gene might not always have sufficient variability to allow for the distinction of closely related isolates or species. More variable genome segments, such as the ITS-1 and ITS-2 regions, might be more useful for that purpose, even if they are too variable to compare distant species (Schnittger et al., 2012). In this sense, our results for these regions corroborate that *B. peircei* and *B. ugwidiensis* are distinct species (with *B. ugwidiensis* infecting both Bank and Cape cormorants), since evolutionary distances for the ITS-1 and ITS-2 regions were

consistently much lower within morphospecies than between morphospecies. The interpretation that these are distinct species is further corroborated by the fact that there are distinguishing morphological characteristics between these species, namely the positioning of merozoite chromatin (proximal in *B. ugwidiensis* and distal in *B. peircei*) (Earlé et al., 1993; Peirce and Parsons, 2012). Of note, *B. ugwidiensis* has also been reported from three other cormorant species from South Africa including the White-breasted cormorant (*P. carbo*), Crowned cormorant (*P. coronatus*), and the Reed cormorant (*P. africanus*). Parasites from these hosts were morphologically identical to *B. ugwidiensis* but we did not have samples to include in our molecular analyses.

The vectors of *B. peircei* and *B. ugwidiensis* are unknown, and both the soft tick *Ornithodoros capensis* and the hard tick *Ixodes uriae* have been speculated to play this role (Earlé et al., 1993; Brossy et al., 1999; Peirce and Parsons, 2012). *O. capensis* is a relatively common parasite of African penguins (Daturi, 1986) as well as of Bank and Cape cormorants (Williams, 1978; Cooper, 1986; Peirce and Parsons, 2012). In contrast, the infestation of these birds by *Ixodes uriae* has never been recorded, but is plausible given that *I. uriae* is known to infest other penguin and cormorant species in other continents and has been documented to occur in the Cape coast of the South Africa (Muñoz-Leal and González-Acuña, 2015). Because African penguins and Bank and Cape cormorants nest in mixed colonies along the southern African coast, it is likely that there are occasional opportunities for exchange of *Babesia*-infected *O. capensis* and/or *I. uriae* from penguins to cormorants and vice-versa. The fact that in spite of such opportunities for cross-transmission we still found considerable ITS-1 and ITS-2 regions sequence differences between *B. peircei* and *B. ugwidiensis* further corroborates that there is reproductive isolation between these parasite species despite the probable overlap in tick vectors.

On the other hand, our results show that the distinction between *Babesia* strains from penguins, gannets, boobies, gulls and terns might be less discernible. Papparini et al. (2014) found that genetically-similar *Babesia* sp. strains infected gannets (Suliformes)

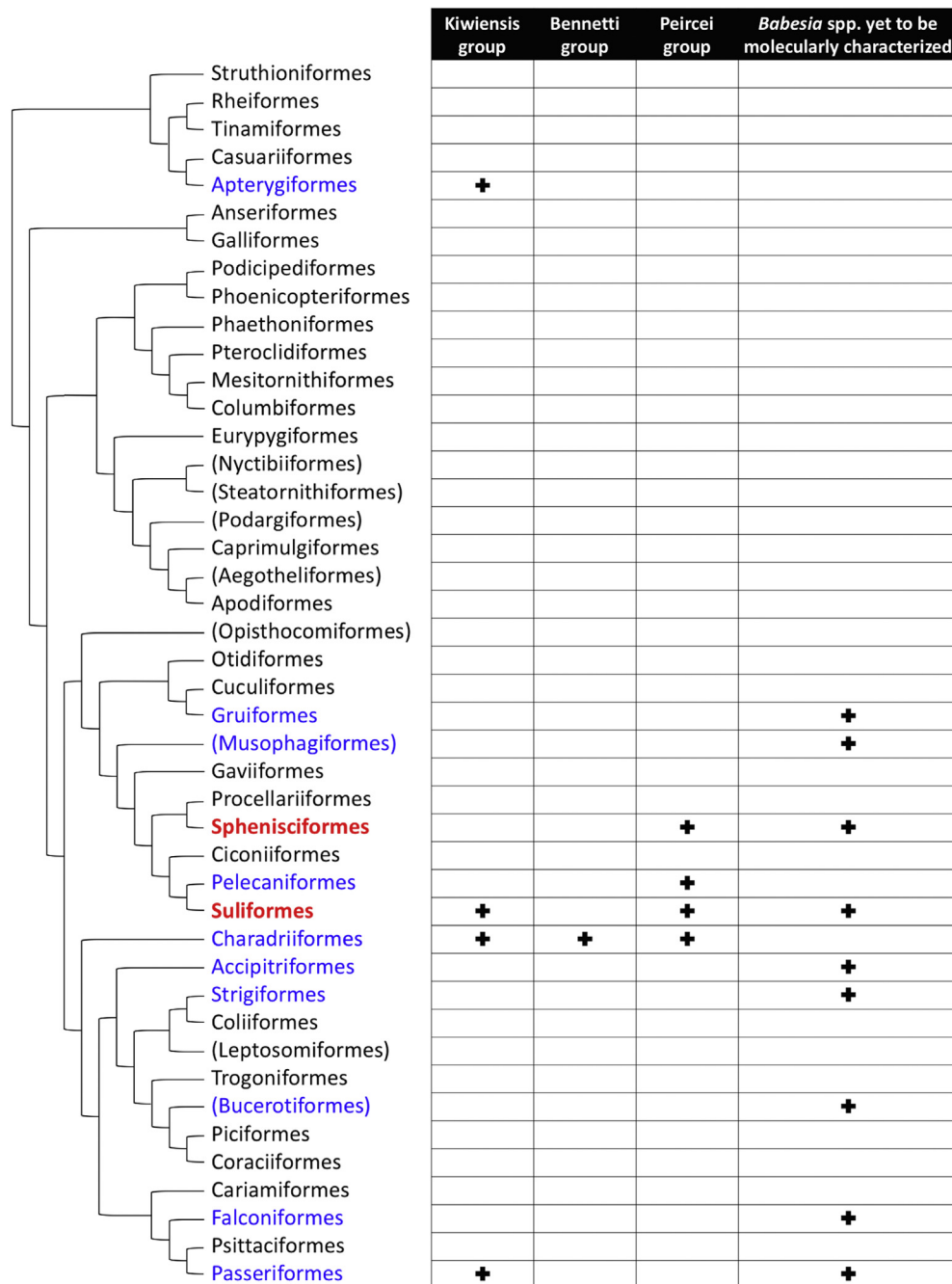


Fig. 3. Distribution of the phylogenetic groups of avian piroplasmids (based on the 18S rRNA gene) in relation to the phylogeny of avian orders (based on multiple nuclear genes). Avian orders investigated in this study are shown in red, and other avian orders known to host piroplasmids are shown in blue. Avian phylogeny was adapted from Yuri et al. (2013). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and gulls and terns (Charadriiformes) in New Zealand, and we found that some of these strains (“genotype 1”) were nearly identical (99.9% identity; 1440/1441 bp) to *Babesia* sp. from little penguins (Sphenisciformes) from Australia (previously reported by Vanstreels et al., 2015). Additionally, the sequences of *B. peircei* in our study were highly similar (99.7%; 1446/1450 bp) to those of *B. poeala* from boobies (Suliformes) from Rocas Atoll (previously reported by Quillfeldt et al., 2014). Our phylogenetic analyses of the ITS-1 and ITS-2 regions also showed that the evolutionary divergence between *B. peircei* and *B. poeala* was relatively low, however the lack of additional *B. poeala* sequences for comparison genes warrants caution in the interpretation of these results.

Considering the evidence for low evolutionary divergence between *B. poeala* and *B. peircei*, along with the remarkable morphological resemblance between them (e.g. both species have the merozoite chromatin positioned on the distal end) (Peirce, 2000), the validity of these as separate species seems questionable, and future studies might conclude that *B. poeala* is in fact a synonym of *B. peircei*. However, we consider the current evidence insufficient for a definitive conclusion, and additional genetic and morphological evidence from a larger number of individuals and locations is warranted before determining whether or not the separation of these taxa is appropriate.

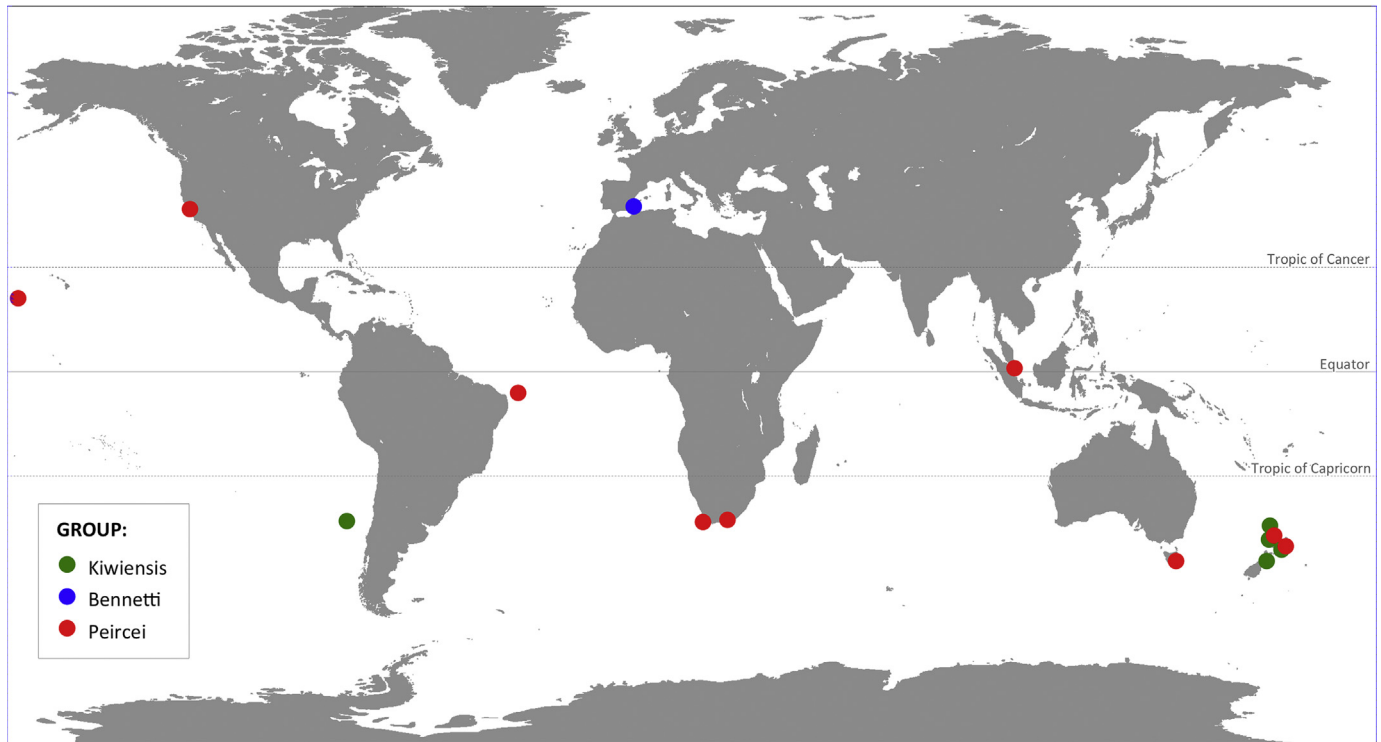


Fig. 4. Geographic distribution of the phylogenetic groups of avian piroplasmids (based on the 18S rRNA gene). Map prepared based on information provided in Criado et al. (2006), Yabsley et al. (2006, 2009), Jefferies et al. (2008), Papparini et al. (2014), Quillfeldt et al. (2014), Martínez et al. (2015), Montero et al. (2016) and Chavatte et al. (2017).

4.3. Gaps of knowledge and directions for the future

Our understanding of the taxonomy, phylogeny and host distribution and specificity of avian piroplasmids is limited. To date, of the 16 described avian *Babesia* species, nine have been reported from only a single host species (Peirce, 2000). In many cases, the original descriptions date back to the 1970s or earlier, and no further studies have examined the same species or regions. Re-description of these parasites in combination with detailed photographs and molecular characterization (e.g., Chavatte et al., 2017) would therefore be valuable to corroborate their taxonomic validity and provide insight into their relationships to other piroplasmid species. Furthermore, additional studies will be necessary to evaluate avian hosts for which piroplasmids have been recorded but were not morphologically characterized in detail, such as barn owls (Mohammed, 1958), great horned owls (*Bubo virginianus*) (Beaufrère et al., 2007), Malagasy paradise flycatchers (*Terpsiphone mutata*) (Savage et al., 2009), Australasian gannets, red-billed gulls, white-fronted terns (Papparini et al., 2014), African darters (*Anhinga rufa*), king penguins (*Aptenodytes patagonicus*), rockhopper penguins (*Eudyptes chrysocome* and *E. moseleyi*), and Cape gannets (*Morus capensis*) (Parsons et al., 2017).

Considering our findings and those of Papparini et al. (2014), it is clear that the identity of avian piroplasmids cannot be assumed based solely on the family or order of their hosts. For instance, all three phylogenetic groups (Kiwiensis, Bennetti and Peircei) have been reported to infect gulls (Charadriiformes: Laridae). Future studies are advised to couple detailed morphological descriptions and measurements with molecular methods to determine the phylogenetic relationships of the parasites. While the 18S rRNA gene should still be considered the gold standard to evaluate phylogenetic relationships for avian piroplasmids, our results illustrate how the analysis of more variable sections of the genome (e.g. ITS-1 and ITS-2 regions) can provide finer scale information on

the phylogeny and taxonomy of closely-related species and might be important in verifying if there is evidence of transmission among multiple hosts. Furthermore, other genes (e.g., beta-tubulin, hsp70, cob, cox1, etc.) may also assist in the evaluation of the phylogenetic relationships and in classifying the parasites more accurately.

Because the ability to conduct controlled experimental infection trials or vector transmission trials is complicated for species such as wild birds, our understanding of the host distribution of avian piroplasmids will benefit from studies on multiple avian species within the same community in order to identify other potentially naturally-infected hosts (e.g., Quillfeldt et al., 2014; Papparini et al., 2014). An aspect that may be particularly relevant for future studies will be investigation of host species-specific variations in the morphology of the parasites, as has long been known to occur in other avian blood parasites (Laird and Van Riper, 1981; Valkiūnas, 2005).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ijppaw.2017.08.006>.

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