Title

Piroplasms of New Zealand seabirds

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Abstract

Blood and ectoparasitic ticks were collected from migratory seabirds in New Zealand, including Australasian gannets (n = 13) from two sites, and red-billed gulls (n = 9) and white-fronted terns (n = 2) from a third location. Blood smears were screened for parasite presence by microscopy, while DNA from blood samples was subjected to PCR for the presence of tick-transmitted protozoan haemoparasites belonging to the order Piroplasmida. Parasites were identified by comparing small subunit ribosomal RNA (18S rDNA) gene sequences to related sequences on GenBank. Analyses indicated that nine birds were infected with unknown variants of a *Babesia poelea*-like parasite (recorded as genotypes I and II), while four harboured a piroplasm that was genetically similar to *Babesia kiwiensis*. There was no parasite stratification by bird species; both the gannets and gulls were positive for all three parasites, while the terns were positive for the *B. kiwiensis*-like and the *B. poelea*-like (genotype I) parasites. The *B. kiwiensis*-like parasite found in the birds was also found in two species of ticks: *Carios capensis* and *Ixodes eudyptidis*. This represents the first report of *Babesia*-positive ticks parasitising seabirds in New Zealand. The lack of host specificity and evidence of wide ranging distributions of the three piroplasm genotypes suggest there is a high degree of haemoparasite transmission occurring naturally between New Zealand seabird populations and species.

Keywords

Piroplasms, *Babesia*, *Theileria*, parasite, wildlife, bird, conservation, phylogeny, systematics

1. Introduction

Arguably, the extraordinary mobility of migratory seabirds, together with their distribution and ability to form very large colonies (with up to millions of individuals in some cases), makes them potentially suitable to spread pathogens of veterinary and medical importance worldwide (Tsiodras, et al., 2008, Wilkinson, et al., 2014). The parasite-vector-host system for migratory seabirds is particularly complex given that the spatial and temporal domains of such interactions are amplified through the hosts biology and ecology (Sandor, et al., 2014).

The order Piroplasmida (phylum: Apicomplexa) includes three main genera of protozoan haemoparasites infecting mammals and birds: *Babesia*, *Theileria* and *Cytauxzoon*. *Babesia* spp. are the second most common tick-transmitted parasites of mammals after the trypanosomes (Hunfeld, et al., 2008), with 16 named species reported from 15 avian families: *B. moshkovskii, B. kazachstanica, B. uriae, B. kiwiensis, B. ardeae, B. frugilegica, B. emberizica, B. shortti, B. balearicae, B. rustica, B. bennetti, B. mujunjumica, B. peircei, B. poelea, B. krylovi*, and *B. ugwidiensis* (Franke, et al., 2010, Jefferies, et al., 2008, Peirce, 2005, Peirce and Parsons, 2012, Yabsley, et al., 2009, Yabsley, et al., 2006, Yabsley, et al., 2006). Additional piroplasms, such as *B. divergens*, *B. venatorum* (formerly *Babesia* sp. EU1 genotype) and *B. microti*, causing bovine and (life-threatening) zoonotic babesiosis respectively, have been identified in ticks removed from migratory birds from various countries (Capligina, et al., 2014, Hasle, et al., 2011, Hildebrandt, et al., 2010, Movila, et al., 2011).

As molecular studies on avian *Babesia* spp. are relatively scarce, small subunit ribosomal RNA gene (18S rDNA) sequences (the main molecular taxonomic marker) are currently available for only four bird-derived *Babesia* parasites: *B. kiwiensis* (GenBank accession number EF551335), *B. poelea* (DQ200887), *B. uriae* (FJ717705) and *B. bennetti* (DQ402155). Sequences from *B. microti*, *B divergens* and *B. venatorum* were also recently obtained (KC297160, KC297161, KC310495; (Capligina, et al., 2014), but their utility for phylogenetic reconstruction is limited by their relatively small length (260-303 base pairs). To improve our knowledge of avian piroplasms, the aim of the present study was to sequence piroplasms in blood sampled from migratory birds in New Zealand. This was carried out as part of a research project, conducted from July 2007 to June 2009, on the ecology of vector-borne diseases in New Zealand and their significance to biosecurity, biodiversity and human health.

1. Materials and Methods

1.1. Sample collection

This work was conducted under DOC (Department of Conservation) Global Concession CA-5160-OTH; DOC Research and Collection Permits NM-22225-RES, ECHB-22299-FAU, AK-22099-FAU, NM-23980-RES and ECHB-24005-FAU; Landcare Research Animal Ethics Authority 07/12/01; New Zealand National Bird Banding Scheme Institutional Permit to Band Birds No. 2007/83; and an Auckland Regional Council permit to undertake research study in Muriwai Regional Park.

Surveys for vector-borne diseases in vertebrate wildlife hosts were carried out in New Zealand during 2008-2009 (Tompkins, et al., 2010). As part of these surveys thin peripheral blood smears, from multiple migratory seabird species, were pre-screened (microscopically) for blood parasites. Intra-erythrocytic inclusions, presumed to be *Babesia* spp., were observed in three hosts: Australasian gannets (*Morus serrator*) from Muriwai Beach and Cape Kidnappers, and red-billed gulls (*Chroicocephalus scopulinus*) and white-fronted terns (*Sterna striata*) from the Kaikoura peninsula (Figure 1).

In the present study, a subset of whole blood samples (n = 24) from individuals of all three species were chosen for follow-up molecular diagnostics (Table 1): eight Australasian gannets from Cape Kidnappers (three with negative blood smears, and five positive for *Babesia* sp.); five Australasian gannets from Muriwai Beach (three blood smears with unidentified inclusions, and two with *Babesia* sp.); nine red-billed gulls (one blood smear with unidentified inclusions and eight with *Babesia* sp.) and two white-fronted terns (both blood smears positive for *Babesia* sp.) from Kaikoura peninsula. A small number of ectoparasitic ticks (n = 8) collected opportunistically at Kaikoura peninsula during the surveys are also tested for piroplasm presence.

1.2. DNA extraction

DNA extractions from blood (0.5 – 1 mL) and ectoparasites were performed in New Zealand, using the QIAamp DNA mini kit (Qiagen, USA). Prior to extractions, ectoparasites were washed twice in sterile, molecular grade water and sliced on sterile Petri dishes using sterile scalpel blades. Mock extractions were also carried out from sterile molecular-grade water, to test for DNA contamination from reagents and consumables. All DNA preparations were checked for

purity by agarose gel electrophoresis and quantified by spectrophotometric absorbance using a Nanodrop ND-1000 (Thermo Scientific, USA). Following extraction, DNA preparations were stored at -20 ºC before shipment to Murdoch University for analysis.

1.3. PCR amplification

DNA samples were screened for the presence of piroplasmid-specific 18S rDNA by nested PCR (850 bp), using the BTF1/BTR1 and BTF2/BTR2 primer pairs as previously described (Jefferies, et al., 2007). A second nested PCR assay, developed during the present study and validated in a previous publication (Paparini, et al., 2012), was also implemented to obtain longer 18S rDNA fragments (1,466 bp) from positive samples.

All PCR reactions (25 µL) were run on a G-Storm GS1 standard block thermal cycler (G-Storm, UK). The nested PCRs were performed using 1 µL of total genomic DNA in the primary PCR, and 1 µL of primary PCR amplicon in the secondary PCR. Reactions included 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.4 µM of each primer, and 0.02 U/µL Kapa Taq DNA polymerase (Kapa Biosystems, USA).

The PCR conditions for the primary PCR (primers BT18SF1/BT18SR1) consisted of a pre-PCR step of 95 ºC for 5 minutes, followed by 40 cycles of 94 ºC for 30 seconds, 52 ºC for 30 seconds, an extension of 72 ºC for 2 minutes, and a final extension of 72 ºC for 7 minutes. The PCR conditions of the secondary PCR (primers BT18SF2/BT18SR2) consisted of a pre-PCR step of 95 ºC for 5 minutes, followed by 40 cycles of 94 ºC for 30 seconds, 50 ºC for 30 seconds, an extension of 72 ºC for 1 minute and 20 seconds, and a final extension of 72 ºC for 7 minutes.

1.4. Molecular identification of avian hosts

A selection of the piroplasmid positive DNA samples were also screened with primers designed to amplify the cytochrome *b* gene of vertebrates, using methods and conditions previously described (Kocher, et al., 1989), to verify vertebrate host identity.

1.5. Sequencing

PCR products were run on a 1% agarose gel containing SYBR Safe Gel Stain (Invitrogen, USA), and visualised with a dark reader trans-illuminator (Clare Chemical Research, USA). Bands corresponding to the expected molecular size were excised from the gel, purified

using an Ultraclean DNA purification Kit (MO BIO, USA), and sequenced bi-directionally (i.e. using both forward and reverse primers) using an ABI Prism Terminator Cycle Sequencing kit (Applied Biosystems, USA) on an Applied Biosystem 3730 DNA Analyzer. Sequences were checked against the GenBank nucleotide database using BLAST, and imported into various sequence manipulation software suites for phylogenetic reconstruction as described below.

1.6. Phylogenetic analysis

Nucleotide sequences generated for the piroplasmid-specific 18S rDNA were aligned with a number of related sequences from GenBank using MUSCLE (Edgar, 2004), and trimmed with Gblocks (Castresana, 2000). Phylogeny was inferred by the maximum likelihood method (ML) using PhyML (Dereeper, et al., 2008), with the GTR (+I (0.56)+G(0.45)) model of evolution (Tavaré, 1986). Reliability of inferred trees was assessed by the approximate likelihood ratio test (aLRT), a statistical test of branch support and an alternative to non-parametric bootstrap branch support estimation (Anisimova and Gascuel, 2006). The appropriate models of nucleotide substitution for ML analysis were chosen using the Akaike information criterion (Akaike, 1974) in the software program jModeltest 0.1.1 (Posada, 2008). Phylogenetic analysis was conducted in MEGA 4 (Tamura, et al., 2007). All codon positions were included, and all positions containing gaps and missing data were eliminated from the dataset (i.e. 'complete deletion' option). Bootstrap analysis was conducted using 1,000 replicates to assess the reliability of inferred tree topologies. The Jukes-Cantor model was chosen following the guidelines of Nei and Kumar (2000), and used with MEGA 4 (Tamura, et al., 2007) to calculate genetic distances.

2. Results

2.1. Ectoparasites

The ticks collected were morphologically identified as *Carios capensis* (formerly *Ornithodoros capensis*; n = 8) and *Ixodes eudyptidis* (n = 5). The *C. capensis* ticks comprised three adult females and five nymphs collected off a red-billed gull; the five *I. eudyptidis* ticks comprised two adult females, two adult males and one nymph collected off another red-billed gull.

2.2. Molecular analyses

Of the 24 blood samples screened by PCR, 64.0 % (n = 16) were positive for piroplasmid-specific 18S rDNA (Table 1): seven gannets (7/13 = 53.8 %), seven gulls (7/9 = 77.8 %), and two terns (2/2 = 100.0 %). Seven ticks, two adult females and four nymphs of *C. capensis,* and one adult male *I. eudyptidis*, were also positive (7/13 = 53.8 %). All positive and negative PCR controls produced appropriate results. Interestingly, there was poor correspondence between those blood samples that were positive on microscopy and those that were PCR positive.

2.3. Phylogenetic analysis

Three PCR positive birds (one gannet and two gulls) produced very faint PCR bands that could not be processed further; sequencing of PCR products was thus only possible for 13/16 positive samples. Phylogenetic reconstruction revealed three new unique sequence variants that grouped with two bird-derived variants already known (Table 2). Two of the new variants were similar to *Babesia poelea* (DQ200887) from brown boobies (Yabsley, et al., 2006), and the third was similar to *Babesia kiwiensis* (EF551335), from the North Island brown kiwi (Jefferies, et al., 2008).

The two nearly-identical *B. poelea*-like sequences (I and II) differed from one another by a single nucleotide polymorphism (SNP) and a single nucleotide insertion/deletion (INDEL) that introduced a gap in the multiple sequence alignment. The *B. poelea*-like sequence I was identified in six birds: three Australasian gannets, two red-billed gulls, and one white-fronted tern. The *B. poelea*-like sequence II was found in three birds: one Australasian gannet and two red-billed gulls. The *B. kiwiensis*-like sequence was found in four birds: two Australasian gannets, one red-billed gull, and one white-fronted tern. The sequences obtained from the PCR positive ticks were all identified as *B. kiwiensis*-like.

The ML phylogenetic analysis of the three piroplasm sequences identified is presented in Figure 2. The *B. poelea*-like isolates obtained during the present study clustered tightly (100 % bootstrap value) with the known *B. poelea*/*B. uriae* clade (Lack, et al., 2012). Despite this, the novel genotypes from the present study appeared significantly different from the known avian species *B. poelea* and *B. uriae*, forming a separate clade with 67 % bootstrap value (*Babesia uriae* has been submitted to GenBank as *Babesia* sp. MJY-2009a; (Yabsley, et al., 2009). An

additional ML phylogenetic analysis was conducted to include *Babesia* sp. isolate JM-2013 identified from brown boobies (*Sula leucogaster*) in Brazil (Quillfeldt, et al., 2011), which has a slightly shorter 18S rDNA sequence. In the resultant ML tree (not presented), *Babesia* sp. isolate JM-2013 also nested within the *B. poelea*/*B. uriae* clade, but separately to the two *B. poelea*-like genotypes (with *B. poelea* from *S. leucogaster* on Johnston Atoll in the Central Pacific; 99 % bootstrap value). Conversely, the *B. kiwiensis*-like sequence obtained during this study grouped with *B. kiwiensis* (99 % bootstrap value), a known species from the North island brown kiwi (*Apteryx australis mantelli*; (Jefferies, et al., 2008).

Pairwise genetic distances showed that the nearly identical *B. poelea*-like sequences obtained here were 0.3±0.2 % and 0.7±0.2 % distant from *B. poelea* and *B. uriae*, respectively, while the *B. kiwiensis*-like sequence obtained here was 1.6±0.3 % distant from *B. kiwiensis* (Table 2).

2.4. Cytochrome *b* PCR

Partial fragments of cytochrome *b* gene were obtained from five of the birds positive for piroplasm DNA (two Australasian gannets, two red-billed gulls, and one white-fronted tern). Although the sequences generated were not 100% readable, they were sufficient to distinguish the three hosts sampled from one another to genus level. The closest GenBank match for the gannet *cyt b* sequences was the northern gannet (*Morus serrator*), for the red-billed gull sequences was the silver gull (*Larus novaehollandiae*), and for the white-fronted tern sequence was confirmed as collected from a white-fronted tern (*Serrator striata*). Apart from a short sequence (GenBank acc. number AF268516), representative cytochrome *b* sequences for the red-billed gull were not available in GenBank.

3. Discussion

A relatively recent review, focusing on avian haemoparasites, concluded that infections probably affect around one third (27 %) of seabirds (Quillfeldt, et al., 2011). In the present study *Babesia* was molecularly identified in Australasian gannets, red-billed gulls and white-fronted terns. To the best of our knowledge, this is the first report of *Babesia* spp. in gannets and terns. Our phylogenetic analysis identified novel minor genetic variants, two of *B. poelea* and one of *B. kiwiensis*. Overall tree topology was consistent with recent published analyses that confirm avian

piroplasms to be more closely related to mammalian *Babesia* spp. than to *Theileria* spp. (Jefferies, et al., 2008, Yabsley, et al., 2009, Yabsley, et al., 2006). *Babesia poelea* was originally assigned to the genus *Babesia* on the basis of lack of observed schizogony in host lymphocytes (Work and Rameyer, 1997). Nonetheless, the exact (molecular) phylogenetic position of the bird piroplasms *B. poelea* and *B. uriae* is problematic (Lack, et al., 2012, Schnittger, et al., 2012). While for Lack et al. (2012) these two species formed a unique, strongly supported clade (≥ 0.95 Bayesian posterior probability and ≥ 70 % maximum parsimony bootstrap value), Schnittger et al. (2012) did not confirm paraphyly and indicated *B. poelea* as a sister species to the whole *B. duncani*/*conradae* clade (the "western clade" or "prototheilerids"). Similarly, in Jefferies et al. (2008) and Yabsley et al. (2006)(Jefferies, et al., 2008, Yabsley, et al., 2006), *B. poelea* clearly clustered with the western clade (93 - 96 % bootstrap value), but also showed a looser association with various *Theileria* spp.

It is well known that the outcome of phylogenetic reconstructions (topology, branch length etc.) can be significantly affected by numerous factors (Brocchieri, 2001). Some of these factors are under the investigator's control (method used, gap treatment option, choice of outgroup, species sampling, alignment strategy etc.), while others are inherent to the dataset and just have to be recognised and processed (alignment quality, species sampling, evolutionary rates, paralogy etc.). With a clear split between the *B. poelea*/*B. uriae* clade and the prototheilerids, the 18S rDNA-based reconstruction presented here is consistent with the results of Lack, et al. (2012). However, all of these studies (including ours) implemented different analytical methods, which may explain some of the observed differences.

Although *B. bennetti* has previously been described in the yellow-legged gull (*Larus cachinnans;* (Merino, 1998), the molecular analyses presented here suggest that this parasite is only distantly related to those detected in gulls here. This finding is consistent with the predictions of previous authors (Lack, et al., 2012, Peirce, 2000), that the true diversity of avian piroplasms will become apparent as more avian piroplasms are described and included in phylogenetic analyses..

Clear-cut criteria have previously been adopted to define the upper/lower limits for genetic identity values, above or below which the corresponding isolates would be affiliated to the same or different species (Schnittger, et al., 2003). For an 18S rDNA dataset, isolates belonging to different *Babesia* species must have similarity values ≤ 96.6% (i.e. genetic distance

≥ 3.4%). In the same way, isolates belonging to a single *Babesia* species have to show ≥ 97.9% similarity (i.e. genetic distance ≤ 2.1%). On the basis of Schnittger's rules, the piroplasmids identified in the present study are insufficiently different at the 18s rDNA gene to *B. kiwiensis* (1.6 % genetic difference) and *B. poelea* (0.3 % genetic difference), to be considered new species. Minor sequence variation can sometimes result from PCR amplification error. This is, however, an unlikely explanation for the sequence variation found in this study, as the genetic differences were not randomly distributed within the sequence but, rather, shared by several individuals.

Babesia uriae was originally described from blood collected from three common murres (also known as common guillemots; *Uria aalge*) from California. It was assigned to the genus *Babesia* and considered a novel species on the basis of i) molecular data and ii) assumed vertebrate host family specificity of avian babesias (Peirce, 2000). This second criterion has also been considered when reviewing the species status of *B. poelea* (Peirce, 2000). Our molecular survey detected *B. kiwiensis*-like and *B. poelea*-like sequences in: Australasian gannets (family *Sulidae*), red-billed gulls (*Laridae*) and white-fronted terns (*Sternidae*). BLAST searches of the sequences obtained from the vertebrate host marker (cytochrome *b*) during the present study confirmed the morphological identifications of the avian species surveyed. In this respect, the present study goes against the hypothesis that most species of (avian) *Babesia* are host-specific to the family and often the sub-family level (Peirce, 2000).

While the molecular association of the bird-derived *B. kiwiensis*-like isolate with other mammalian babesids confirms previous studies (Jefferies, et al., 2008, Yabsley, et al., 2009, Yabsley, et al., 2006), it potentially conveys misleading information. The molecular systematics of avian piroplasms (18S rDNA) currently relies on sequences from only four taxa: *B. kiwiensis* (EF551335), *B. poelea* (DQ200887), *B. uriae* (FJ717705) and *B. bennetti* (DQ402155). Such limited sequence data available from wildlife may have led, for example, to deceivingly close phylogenetic associations of *Babesia* sp. FP44 from the Florida panther (DQ329138) with *Babesia* sp. AJB-2006 from the North American river otter (DQ028958) and *Babesia* sp. MA#230 from a Japanese raccoon (AB251608); the genetic distances between these piroplasmids is ≤ 2.9 ± 0.5 %; (Jinnai, et al., 2009)Figure 2). It is expected that piroplasms systematics will change as we discover more information, to provide increasingly better approximates of the true phylogeny of the whole group.

The *Babesia* sequences identified here showed no evidence of host specificity. This also suggests a low specificity of the tick vector(s) for the families of seabirds sampled (*Sulidae*, *Laridae* and *Sternidae*), a suggestion which is compatible with high observed global tick infestation rates of seabirds (Dietrich, et al., 2011). In New Zealand, at least seven tick can infest seabirds (*C. capensis*, *I. amersoni*, *I. auritulus zealandicus*, *I. eudyptidis*, *I. jacksoni*, *I. kerguelenensis*, and *I. uriae*; (Heath, et al., 2011)), but no piroplasm species has ever been detected in seabird ticks (Dietrich, et al., 2011). The present study identified piroplasmid-specific DNA (*B. kiwiensis*-like) in two species of ticks: *C. capensis* and *I. eudyptidis*. This thus represents the first report of babesia-positive seabird ticks from New Zealand. Since ticks are haematophagous, it is not unexpected that identical piroplasm sequences were identified in both vertebrate- and invertebrate hosts; despite the fact that vector competency cannot be confirmed, this finding suggests that these two tick species are vectors of the *Babesia* spp. detected.

Our analysis of samples from seabirds from three diverse locations in New Zealand suggests the possibility of inter-host species transmission and shows spatial dispersal of piroplasmids in different NZ seabird populations. Although this raises questions about the importance of these migratory birds as potential reservoirs of clinically- and veterinary-relevant parasites (and about the role of these birds in their dissemination), no known zoonotic species were found during the present survey. The detection of DNA belonging to avian piroplasmids in ticks associated with these wild birds also suggests the potential role for these ectoparasites as vectors, with potential implications for possible tick-management strategies.

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References

Akaike, H., 1974. A new look at the statistical model identification. IEEE Transactions on Automatic Control 19**,** 716-723.

Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. Systematic Biology 55**,** 539-552.

Brocchieri, L., 2001. Phylogenetic inferences from molecular sequences: Review and critique. Theor Popul Biol 59**,** 27-40.

Capligina, V., Salmane, I., Keiss, O., Vilks, K., Japina, K., Baumanis, V., Ranka, R., 2014. Prevalence of tick-borne pathogens in ticks collected from migratory birds in Latvia. Ticks Tick Borne Dis 5**,** 75-81.

Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17**,** 540-552.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.M., Gascuel, O., 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Research 36**,** W465-469.

Dietrich, M., Gomez-Diaz, E., McCoy, K.D., 2011. Worldwide distribution and diversity of seabird ticks: implications for the ecology and epidemiology of tick-borne pathogens. Vector-Borne and Zoonotic Diseases 11**,** 453-470.

Edgar, R.C., 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5**,** 113.

Franke, J., Meier, F., Moldenhauer, A., Straube, E., Dorn, W., Hildebrandt, A., 2010. Established and emerging pathogens in Ixodes ricinus ticks collected from birds on a conservation island in the Baltic Sea. Med Vet Entomol 24**,** 425-432.

Hasle, G., Leinaas, H.P., Roed, K.H., Oines, O., 2011. Transport of Babesia venatorum-infected Ixodes ricinus to Norway by northward migrating passerine birds. Acta Vet Scand 53.

Heath, A.C.G., Palma, R.L., Cane, R.P., Hardwick, S., 2011. Checklist of New Zealand ticks (Acari: Ixodidae, Argasidae). Zootaxa**,** 55-63.

Hildebrandt, A., Franke, J., Meier, F., Sachse, S., Dorn, W., Straube, E., 2010. The potential role of migratory birds in transmission cycles of Babesia spp., Anaplasma phagocytophilum, and Rickettsia spp. Ticks Tick Borne Dis 1**,** 105-107.

Hunfeld, K.P., Hildebrandt, A., Gray, J.S., 2008. Babesiosis: Recent insights into an ancient disease. Int J Parasitol 38**,** 1219-1237.

Jefferies, R., Down, J., McInnes, L., Ryan, U., Robertson, H., Jakob-Hoff, R., Irwin, P., 2008. Molecular characterization of *Babesia kiwiensis* from the brown kiwi (*Apteryx mantelli*). J Parasitol 94**,** 557-560.

Jefferies, R., Ryan, U.M., Irwin, P.J., 2007. PCR-RFLP for the detection and differentiation of the canine piroplasm species and its use with filter paper-based technologies. Vet Parasitol 144**,** 20-27.

Jinnai, M., Kawabuchi-Kurata, T., Tsuji, M., Nakajima, R., Fujisawa, K., Nagata, S., Koide, H., Matoba, Y., Asakawa, M., Takahashi, K., Ishihara, C., 2009. Molecular evidence for the presence of new *Babesia* species in feral raccoons (*Procyon lotor*) in Hokkaido, Japan. Vet Parasitol 162**,** 241-247.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F.X., Wilson, A.C., 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Natl Acad Sci U S A 86**,** 6196-6200.

Lack, J.B., Reichard, M.V., Van Den Bussche, R.A., 2012. Phylogeny and evolution of the Piroplasmida as inferred from 18S rRNA sequences. Int J Parasitol 42**,** 353-363.

Merino, S., 1998. Babesia bennetti n. sp. from the Yellow-Legged Gull (*Larus cachinnans*, Aves, Laridae) on Benidorm Island, Mediterranean Sea. The Journal of Parasitology 84**,** 422-424.

Movila, A., Reye, A.L., Dubinina, H.V., Tolstenkov, O.O., Toderas, I., Huebschen, J.M., Muller, C.P., Alekseev, A.N., 2011. Detection of Babesia Sp. EU1 and Members of Spotted Fever Group Rickettsiae in Ticks Collected from Migratory Birds at Curonian Spit, North-Western Russia. Vector-Borne and Zoonotic Diseases 11**,** 89-91.

Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, Inc., New York.

Paparini, A., Ryan, U.M., Warren, K., McInnes, L.M., de Tores, P., Irwin, P.J., 2012. Identification of novel *Babesia* and *Theileria* genotypes in the endangered marsupials, the woylie (*Bettongia penicillata ogilbyi*) and boodie (*Bettongia lesueur*). Exp Parasitol 131**,** 25-30.

Peirce, M.A., 2000. A taxonomic review of avian piroplasms of the genus *Babesia* Starcovici, 1893 (Apicomplexa: Piroplasmorida: Babesiidae). Journal of Natural History 34**,** 317-332.

Peirce, M.A., 2005. A checklist of the valid avian species of Babesia (Apicomplexa : Piroplasmorida), Haemoproteus, Leucocytozoon (Apicomplexa : Haemosporida), and Hepatozoon (Apicomplexa : Haemogregarinidae). Journal of Natural History 39**,** 3621-3632.

Peirce, M.A., Parsons, N.J., 2012. *Babesia ugwidiensis*, a new species of avian piroplasm from Phalacrocoracidae in South Africa. Parasite-Journal De La Societe Francaise De Parasitologie 19**,** 375-379.

Posada, D., 2008. jModelTest: Phylogenetic Model Averaging. Mol Biol Evol 25**,** 1253-1256.

Quillfeldt, P., Arriero, E., Martinez, J., Masello, J.F., Merino, S., 2011. Prevalence of blood parasites in seabirds - a review. Frontiers in Zoology 8.

Sandor, A.D., Marcutan, D.I., D'Amico, G., Gherman, C.M., Dumitrache, M.O., Mihalca, A.D., 2014. Do the Ticks of Birds at an Important Migratory Hotspot Reflect the Seasonal Dynamics of Ixodes ricinus at the Migration Initiation Site? A Case Study in the Danube Delta. PLoS ONE 9.

Schnittger, L., Rodriguez, A.E., Florin-Christensen, M., Morrison, D.A., 2012. Babesia: A world emerging. Infect Genet Evol 12**,** 1788-1809.

Schnittger, L., Yin, H., Gubbels, M., Beyer, D., Niemann, S., Jongejan, F., Ahmed, J., 2003. Phylogeny of sheep and goat *Theileria* and *Babesia* parasites. Parasitol Res 91**,** 398-406.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24**,** 1596-1599.

Tavaré, S., 1986. Some Probabilistic and Statistical Problems in the Analysis of DNA Sequences, American Mathematical Society: Lectures on Mathematics in the Life Sciences. Amer Mathematical Society, pp. 57-86.

Tompkins, D.M., Paterson, R., Massey, B., Gleeson, D.M., 2010. Whataroa virus four decades on: emerging, persisting, or fading out? Journal of the Royal Society of New Zealand 40**,** 1-9.

Tsiodras, S., Kelesidis, T., Kelesidis, I., Bauchinger, U., Falagas, M.E., 2008. Human infections associated with wild birds. J Infect 56**,** 83-98.

Wilkinson, D.A., Dietrich, M., Lebarbenchon, C., Jaeger, A., Le Rouzic, C., Bastien, M., Lagadec, E., McCoy, K.D., Pascalis, H., Le Corre, M., Dellagi, K., Tortosa, P., 2014. Massive Infection of Seabird Ticks with Bacterial Species Related to Coxiella burnetii. Appl Environ Microbiol 80**,** 3327-3333.

Work, T.M., Rameyer, R.A., 1997. Description and epizootiology of *Babesia poelea* n. sp. in brown boobies (Sula leucogaster (Boddaert)) on Sand Island, Johnston Atoll, Central Pacific. J Parasitol 83**,** 734-738.

Yabsley, M.J., Greiner, E., Tseng, F.S., Garner, M.M., Nordhausen, R.W., Ziccardi, M.H., Borjesson, D.L., Zabolotzky, S., 2009. Description of novel *Babesia* species and associated lesions from common murres (*Uria aalge*) from California. J Parasitol 95**,** 1183-1188.

Yabsley, M.J., Murphy, S.M., Cunningham, M.W., 2006. Molecular detection and characterization of *Cytauxzoon* felis and a *Babesia* species in cougars from Florida. J Wildl Dis 42**,** 366-374.

Yabsley, M.J., Work, T.M., Rameyer, R.A., 2006. Molecular phylogeny of *Babesia poelea* from brown boobies (*Sula leucogaster*) from Johnston Atoll, Central Pacific. J Parasitol 92**,** 423-425.

Figure caption

Figure 1. During surveys for vector-borne diseases in vertebrate wildlife hosts carried out 2008-9 in New Zealand, thin peripheral blood smears were collected from multiple migratory seabird species (Tompkins, et al., 2010). *Babesia* spp. were identified microscopically in three hosts: Australasian gannets from Muriwai Beach and Cape Kidnappers, and red-billed gulls and white-fronted terns from Kaikoura peninsula. DNA extracted from whole blood samples from all three species (and associated ectoparasitic ticks from Kaikoura peninsula) was analysed molecularly during the present study.

Figure 2. Phylogenetic analysis of the Piroplasmida, based on partial sequences of the 18S rDNA locus. Evolutionary history was inferred using the Maximum Likelihood method. Branch support/bootstrap values (≥50 % only shown) are indicated by each node. Wild seabird-derived sequences obtained from the present study are in bold, and identified by the triangle (▼). Known closely related taxa already available from GenBank are in bold.

Table 1
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Table 1. Summary of the bird blood samples tested for piroplasm-specific 18S rDNA, and sequence type obtained. Samples IDs and blood smear results are from Tompkins et al. (2010).

Vertebrate (avian) host		Sampling location	Microscopic identification	Molecular identification
Bird field ID	**Species (common name)**		**Blood smear**	**DNA**
08-109 #8	Australasian gannet	Cape Kidnappers (n=8)	Negative	*B. poelea*-like I
08-109 #15	Australasian gannet		Negative	*B. poelea*-like I
08-122 #36	Australasian gannet		Negative	*B. poelea*-like II
09-1454 #2	Australasian gannet		*Babesia* sp.	Unknown positive
09-1454 #3	Australasian gannet		*Babesia* sp.	*B. poelea*-like I
09-1454 #4	Australasian gannet		*Babesia* sp.	Negative
09-1454 #11	Australasian gannet		*Babesia* sp.	Negative
09-1454 #22	Australasian gannet		*Babesia* sp.	Negative
08-165 #1	Australasian gannet	Muriwai beach (n=5)	Unidentified inclusion	*B. kiwiensis*-like
08-165 #11	Australasian gannet		Unidentified inclusion	Negative
08-167 #1	Australasian gannet		Unidentified inclusion	Negative
08-229 #18	Australasian gannet		*Babesia* sp.	*B. kiwiensis*-like
08-229 #19	Australasian gannet		*Babesia* sp.	Negative
08-231 #4	Red-billed gull	Kaikoura peninsula (n=11)	Unidentified inclusion	Negative
08-231 #8	Red-billed gull		*Babesia* sp.	*B. kiwiensis*-like
08-231 #17	Red-billed gull		*Babesia* sp.	*B. poelea*-like I
08-2409 #114	Red-billed gull		*Babesia* sp.	*B. poelea*-like II
08-2409 #191	Red-billed gull		*Babesia* sp.	*B. poelea*-like I
08-2409 #206	Red-billed gull		*Babesia* sp.	Negative
08-2409 #221	Red-billed gull		*Babesia* sp.	Unknown positive
08-2409 #222	Red-billed gull		*Babesia* sp.	*B. poelea*-like II
08-2409 #223	Red-billed gull		*Babesia* sp.	Unknown positive
09-1454 #549	White-fronted tern		*Babesia* sp.	*B. kiwiensis*-like
09-1454 #590	White-fronted tern		*Babesia* sp.	*B. poelea*-like I
		Total positive identifications	***17***	***13***
		Uncertain identifications	***4***	***3***

Table 2
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Table 2. Percentage pairwise genetic distances ± standard error (S.E.) between the 18S rDNA of the piroplasmids from Australasian gannets, Red-billed gulls and White-fronted terns (in bold), and the most closely related known piroplasms. Distances were determined using the Jukes-Cantor nucleotide substitution model. GenBank accession numbers for previously known species are given in brackets. Minimum pairwise distance values, between each genotype from the present study and known matches present in GenBank, are shaded for convenience.

	***B. kiwiensis*-like**	***B. poelea*-like genotype I**	***B. poelea*-like genotype II**	*B. kiwiensis* (EF551335)	*B. poelea* (DQ200887)
***B. poelea*-like genotype I**	6.3±0.7				
***B. poelea*-like genotype II**	6.3±0.7	0.0±0.0			
B. kiwiensis (EF551335)	1.6±0.3	6.7±0.7	6.7±0.7		
B. poelea (DQ200887)	6.6±0.7	0.3±0.2	0.3±0.2	6.9±0.7	
B. uriae[*] (FJ717705)	6.6±0.7	0.7±0.2	0.7±0.2	7.0±0.7	0.6±0.2

[*]*Babesia uriae* was originally submitted to GenBank as *Babesia* sp. MJY-2009a.

Figure 1
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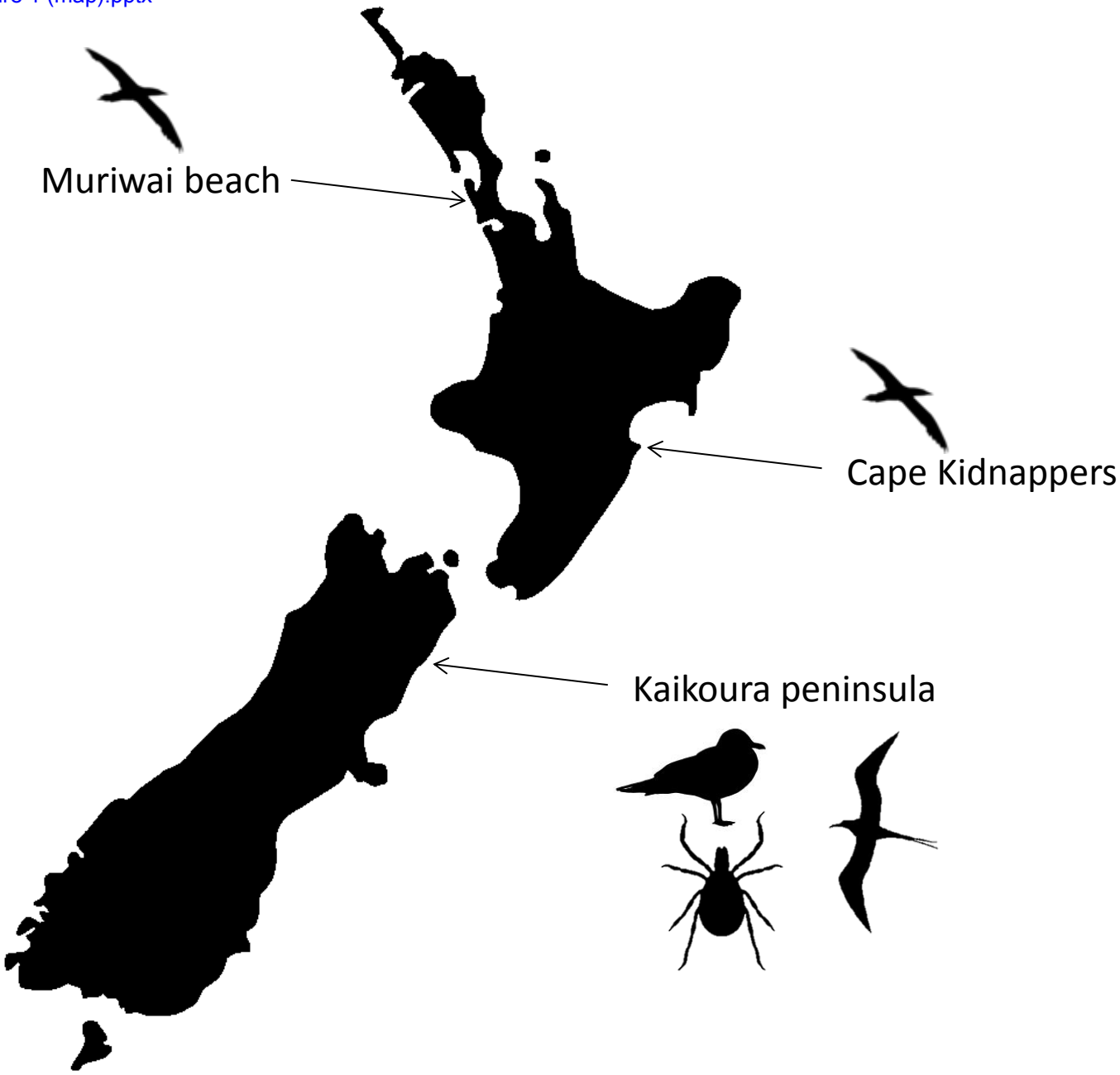

Figure 2

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