Molecular Phylogeny of *Babesia poelea* From Brown Boobies (*Sula leucogaster*) From Johnston Atoll, Central Pacific

Michael J. Yabsley, Thierry M. Work*, **and Robert A. Rameyer***, Southeastern Cooperative Wildlife Disease Study and Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602. *e-mail: myabsley@uga.edu*; * U.S. Geological Survey, National Wildlife Health Center, Honolulu Field Station, P.O. Box 50167, Honolulu, Hawaii 96850

ABSTRACT: The phylogenetic relationship of avian *Babesia* with other piroplasms remains unclear, mainly because of a lack of objective criteria such as molecular phylogenetics. In this study, our objective was to sequence the entire 18S, ITS-1, 5.8S, and ITS-2 regions of the rRNA gene and partial ß-tubulin gene of *B. poelea*, first described from brown boobies (*Sula leucogaster*) from the central Pacific, and compare them to those of other piroplasms. Phylogenetic analyses of the entire 18S rRNA gene sequence revealed that *B. poelea* belonged to the clade of piroplasms previously detected in humans, domestic dogs, and wild ungulates in the western United States. The entire ITS-1, 5.8S, ITS-2, and partial ß-tubulin gene sequence shared conserved regions with previously described *Babesia* and *Theileria* species. The intron of the ß-tubulin gene was 45 bp. This is the first molecular characterization of an avian piroplasm.

Piroplasms are one of the most common parasites of mammals. Many species of piroplasms can cause severe disease in their mammalian hosts and thus are of considerable medical and veterinary importance (Homer et al., 2000). The majority of piroplasms have been described based entirely on morphology and/or host. However, morphology and host species often are insufficient to identify them to species because multiple species of morphologically identical species can infect the same host, or multiple host species may be infected with a single piroplasm species (Kjemtrup, Kocan et al., 2000; Holman et al., 2003). The recent use of molecular tools has clarified the classification of mammalian piroplasms into 4 primary groups, i.e., the *Babesia* species sensu stricto, the *Theileria* and *Cytauxzoon* species, the western clade piroplasms, and *Babesia microti* and related small babesiae (Kjemtrup, Kocan et al., 2000; Kjemtrup, Thomford et al., 2000). The *Babesia* species sensu stricto clade has been subdivided further into the babesids and the ungulibabesids (Criado-Fornelio et al., 2003).

The taxonomic status of the avian piroplasms has been confusing because all species have been described based on morphology and either assigned to various genera (*Babesia*, *Nuttallia*, *Sogdianella*, and *Babesiosoma*) or confused with *Plasmodium*, *Haemoproteus*, or *Aegyptianella* (Peirce, 2000). Peirce (1975) transferred all 14 described avian piroplasms from 14 avian families to *Babesia*.

Morphologically, the avian piroplasms resemble red blood stages of *Babesia*, *Theileria*, and *Cytauxzoon*. The avian *Babesia* are characterized by intracytoplasmic erythrocytic development, lack of hemozoin pigment, presence of ring and amoeboid forms, development of fan-shaped or cruciform tetrad schizonts, and lack of exoerythrocytic schizogony. The absence of exoerythrocytic schizonts in avian *Babesia* argued against their inclusion in *Theileria* or *Cytauxzoon* (Peirce, 2000). Schurenkova (1938) reported schizonts in peripheral leucocytes and reticuloendothelial cells of *B. moshkovskii* from vultures (*Gypaetus barbatus*); however, Laird and Lari (1957) thought these structures were host chromatin. No schizonts were seen when *B. moshkovskii* was redescribed from the griffon vulture (*Gyps fulvus*) (Merino et al., 2002). Similarly, histologic examination of tissues and blood smears from birds infected with other *Babesia* species (including *B. poelea*) has also not revealed any evidence of exoerythrocytic schizonts (Work and Rameyer, 1997; Peirce, 2000).

The phylogenetic relationship of avian *Babesia* to mammalian piroplasms remains unclear, mainly because of a lack of additional objective criteria such as molecular phylogenetics, to justify the present classification in *Babesia*. Our objective was to sequence the entire 18S, ITS-1, 5.8S, and ITS-2 regions of the rRNA gene and a fragment of the ß-tubulin gene of *B. poelea* and compare these sequences to those of other

piroplasms. *Babesia poelea* was first described from brown boobies (*Sula leucogaster*) from Sand Island, Johnston Atoll, central Pacific (Work and Rameyer, 1997). A morphologically similar parasite has been detected in masked boobies (*Sula dactylatra melanops*) from Desnoeufs Island, Amirantes, Indian Ocean (Peirce and Feare, 1978).
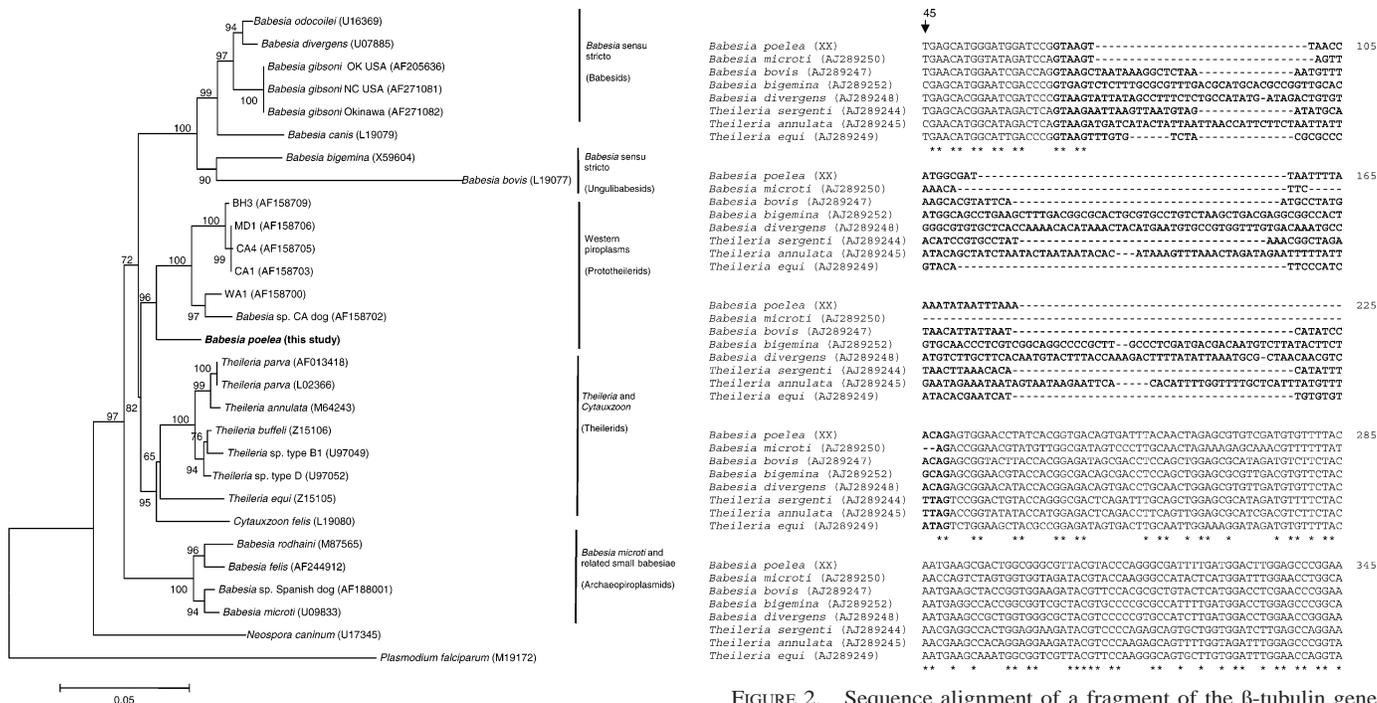
Blood samples from brown boobies were collected at Johnston Atoll National Wildlife Refuge (16°45′N, 169°31′W) located approximately 1,154 km southwest of Honolulu, Hawaii. Juvenile birds were captured in July 1995 using hand nets, and blood was collected from the cutaneous ulnar vein into EDTA. Blood smears were stained with Wright's Giemsa (Difquick; Fisher Scientific, Pittsburgh, Pennsylvania), and parasites quantified (Work and Rameyer, 1997). Remaining blood was frozen at −20 C. For this study, samples from the 3 birds with the highest *B. poelea* parasitemias (~2% each) were selected for DNA extraction and phylogenetic analyses.

For PCR, DNA was extracted from 10 µl of whole blood 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* 18S rRNA gene was done using 5 µl of DNA in a 25 µl reaction containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Promega, Madison, Wisconsin), 2.5 units Taq DNA Polymerase (Promega), and 0.8 µM of primers 5.1 (5′-CCTGGTTGATCCTGCCAGTAGT) and B (Medlin et al., 1989). Cycling parameters for the primary amplification was 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 (5′-CATATGCTTGTCTTAAA) and 3.1 (5′-CTCCTTCCTTTAAGT-GATAAG) or F and R (Gubbels et al., 1999) were used. Cycling conditions were the same as primary reaction except the annealing temperature was 52 C or 50 C, respectively. The entire ITS1, 5.8S, ITS2, and partial 28S rRNA genes were amplified as previous described using primers 1055F and ITSR in a primary reaction and the primers ITSF and LSUR300 in a secondary reaction (Holman et al., 2003).

Primary outside amplification of the ß-tubulin gene was done using 5 µl of DNA in a 25 µl reaction containing the same PCR components as the 18S rRNA PCR except for the use of primers F34 and R323 (Caccio et al., 2000). 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 F79 and R206 were used (Caccio et al., 2000). Cycling parameters for both primary and secondary reactions were 94 C for 5 min followed by 35 cycles of 94 C for 30 sec, 62 C for 30 sec, and 72 C for 1 min (Caccio et al., 2000).

To prevent contamination, DNA extraction, primary and secondary amplification, and product analysis were done in separate dedicated areas. Two negative water controls were included in each set of DNA extractions, and 1 water control was included in each set of primary and secondary PCR reactions. Representative products were purified with a Microcon spin filter (Amicon Inc., Beverly, Massachusetts), sequenced at MWG-BIOTECH (High Point, North Carolina), and sequences compared to those of GenBank.

Sequences obtained from this study and from other piroplasms stored in GenBank (Fig. 1) were aligned using the multisequence alignment ClustalX program (Thompson et al., 1994). Phylogenetic analyses were conducted using the MEGA (Molecular Evolutionary Genetics Analysis) version 3.1 program (Kumar et al., 2004) with the neighbor-joining algorithm using the Kimura 2-parameter model and maximum parsimony using a heuristic search. The GenBank accession numbers for the

FIGURE 1.   Phylogenetic tree inferred by neighbor-joining analysis using the Kimura 2-parameter model of full-length 18S rRNA gene sequences among *Babesia* spp., *Theileria* spp., *Cytauxzoon felis*, unclassified piroplasms (western piroplasms), and *Plasmodium falciparum* (outgroup). The percentages of 1,000 bootstrap replications in which groupings appeared are noted above the branches. Major clade names are shown to right of tree (clade names as suggested by Criado-Fornelio et al. [2003] are shown in parentheses).

FIGURE 2.   Sequence alignment of a fragment of the ß-tubulin gene from *B. poelea* and related piroplasms. Nucleotide position is based on the *B. microti* (AJ289250) sequence. Gaps (—) represent spaces introduced to maximize the homology between aligned sequences. Asterisks (*) below nucleotides indicated conserved positions. The intron region is shown in bold.

18S, ITS-1, 5.8S, and ITS-2 regions of the rRNA and ß-tubulin gene sequences of *B. poelea* are DQ200887 and DQ200888, respectively.

A 1,764 bp amplicon was amplified and sequenced from a single sample of *B. poelea* using the nested protocol (primers 5.1/B and KjF/3.1). A 464 bp amplicon was amplified and sequenced from 2 additional *B. poelea*-positive samples using the nested PCR protocol (5.1/B and F/R); the sequence of these amplicons was identical to the large amplicon. Alignment of the entire 18S rRNA gene sequence of *B. poelea* with related piroplasms and *P. falciparum* (as outgroup, M19172) resulted in an alignment 2,082 bp in length, of which 1,070 were invariant, 306 variable characters were parsimony uninformative, and 416 were parsimony informative.

Both neighbor-joining and maximum parsimony analyses placed *B. poelea* in the clade that included the piroplasms reported from wildlife, humans, and domestic dogs (previously designated *B. gibsoni*) from the western United States (Kjemtrup, Kocan et al., 2000; Kjemtrup, Thomford et al., 2000) (Fig. 1). Both analyses produced trees with similar topology and the same major clades of piroplasms previously reported (Kjemtrup, Kocan et al., 2000; Kjemtrup, Thomford et al., 2000; Goethert and Telford, 2003; Criado-Fornelio et al., 2003), but the separation of the western piroplasm and *Theileria/Cytauxzoon* clades in the maximum parsimony analysis had lower bootsrap support (66%) (data not shown). The highest percent identity to *B. poelea* was observed for the western piroplasms from humans, mule deer, and bighorn sheep (90.6–90.9%), followed by the *Theileria* species (90.0–90.2%).

Based on sequence analysis, the ITS-1, 5.8S, and ITS-2 regions were 421 bp, 154 bp, and 281 bp in length, respectively. All 3 gene sequences were determined for 2 samples of *B. poelea* and were found to be identical for each bird sample. Little identity was shared between *B. poelea* and other piroplasms (available in GenBank) in the ITS-1 region (30.7–40.1%) and the ITS-2 region (41.3–50%). Limited 28S rRNA gene sequences for piroplasms are available for comparison with the

partial 28S rRNA gene sequence (221 bp) determined for *B. poelea* in this study.

Amplification of a fragment of the ß-tubulin gene of *B. poelea* resulted in a 182 bp amplicon that had several conserved regions with other *Babesia* and *Theileria* species (Fig. 2). An intron of 45 bp was present in *B. poelea*; this intron previously has been shown to be variable in size between piroplasm species (Caccio et al., 2000). *Babesia poelea* had similar sequence identity to both *Babesia* species (68.6–81.8%) and *Theileria* species (68.6–73.7%); however, no sequences for the ß-tubulin exon region are available for the western piroplasms.

This study is the only extant molecular characterization of an avian piroplasm and reveals that *B. poelea* is most closely related to the western clade of piroplasms, a group distinct from other piroplasms (Kjemtrup, Kocan et al., 2000; Kjemtrup, Thomford et al., 2000). The inclusion of *B. poelea* within the western piroplasm clade was well supported by both phylogenetic analyses. The western clade includes piroplasms from humans, mule deer, fallow deer, bighorn sheep, and domestic dogs (Kjemtrup, Kocan et al., 2000; Kjemtrup, Thomford et al., 2000). The western piroplasms initially were believed to be more closely related to the *Theileria/Cytauxzoon* spp. (Kjemtrup, Thomford et al., 2000), but later analyses including additional species failed to support this relationship and produced a tricotomy with the *Babesia* sensu strico (Kjemtrup, Kocan et al., 2000). One of our analyses (neighbor joining) provided good support for a sister relationship between the western piroplasms and the *Theileria/Cytauxzoon* group, but this relationship had less support in the maximum parsimony analysis. However, the western piroplasm clade is clearly distinct from the other groups of piroplasms, and the generic status of the western piroplasms has not been determined and may represent a new genus or family (Criado-Fornelio et al., 2003). Additional sequences from other avian piroplasms or closely related piroplasms in the future may show that *B. poelea* branches off to form a separate clade.

Analysis of the partial ß-tubulin gene supported the findings of the rRNA gene sequences: *B. poelea* is a piroplasm related to the genera *Babesia* and *Theileria*. The highest similarity was observed between *B. poelea* and the *Babesia* spp. sensu stricto (*B. divergens* and *B. bigemina*) group; however, only a short exon sequence was available for

analysis. Additional sequence information would be more informative, but attempts to amplify a larger sequence using other sets of published primers (Goethert and Telford, 2003; Zamoto et al., 2004) have failed (M. Yabsley, unpubl. obs.).

This study included only a single *Babesia* species from 3 individuals of the brown booby from the same geographic area. Future studies that include additional avian *Babesia* would help clarify the relationships of avian to mammalian piroplasms. Many avian *Babesia* have been described based on an assumed host family specificity, despite overlap of several morphologic characters, e.g., size of ring form and morphology of merozoites (Pierce, 2000). Because of the difficulties in conducting cross-transmission studies to investigate host specificity, molecular studies, such as the present study, can be used to investigate host specificity and the diversity of avian piroplasms as done for mammals (Goethert and Telford, 2003). Brown boobies are found throughout the world in tropical oceans, but *B. poelea* has been reported only from the brown booby population on Johnston Atoll in the Pacific Ocean, although infections have been detected in brown boobies from the island of Oahu, Hawaii (T. Work, unpubl. obs.). Molecular analyses of avian piroplasms from other locations and hosts are needed to better understand the phylogeny of this intriguing group of parasites.

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