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Description of *Babesia coryicola* sp. nov. from Florida pumas (*Puma concolor coryi*) from southern Florida, USA

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ABSTRACT

Previously, a high prevalence of piroplasms has been reported from Florida pumas (*Puma concolor coryi)* from southern Florida. In the current study, we describe the biological characteristics of a novel *Babesia* species in Florida pumas. Ring-stage trophozoites were morphologically similar to trophozoites of numerous small babesids of felids including *B. leo, B. felis*, and *Cytauxzoon felis*. Parasitemias in Florida pumas were very low (<1%) and hematologic values of 25 *Babesia*-infected Florida pumas were within normal ranges for *P. concolor*. Phylogenetic analysis of near full-length 18S rRNA gene, β-tubulin, cytochrome *c* oxidase subunit I, cytochrome *c* oxidase subunit III, and cytochrome *b* gene sequences indicated that this *Babesia* species is a member of the *Babesia* sensu stricto clade and is related to groups of *Babesia* spp. from carnivores or ungulates, although the closest group varied by gene target. Internal transcribed spacer (ITS)-1 region sequences from this *Babesia* sp. from 19 Florida pumas were 85.7–99.5% similar to each other and ~88% similar to *B. odocoilei.* Similarly, an ITS-2 sequence from one puma was 96% similar to *B. bigemina* and 92% similar to a *Babesia* sp. from a red panda (*Ailurus fulgens*). Infected pumas were positive for antibodies that reacted with *B. odocoilei*, *B. canis,* and *B. bovis* antigens with titers of 1:256, 1:128, and 1:128, respectively. No serologic reactivity was noted for *Theileria equi*. No molecular evidence of congenital infection was detected in 24 kittens born to 11 *Babesia*-infected female pumas. Pumas from other populations in the United States [Louisiana (n = 1), North Dakota (n = 5) and Texas (n = 28)], British Columbia, Canada (n = 9), and Costa Rica (n = 2) were negative for this *Babesia* sp. Collectively, these data provide morphologic, serologic, genetic, and natural history data for this novel *Babesia* sp. which we propose the name *Babesia* coryicola sp. nov. sp. This is the first description of a felid-associated *Babesia* species in North America.

1. Introduction

Florida pumas (*Puma concolor coryi)* are an endangered population of *P. concolor* which has persisted in southern Florida despite the extirpation of *P. concolor* from the rest of eastern North America. Because of decreased genetic diversity and evidence of inbreeding depression, eight female pumas from western Texas were introduced into the range of the Florida pumas in 1995, five of which produced offspring (Johnson et al., 2010). This genetic introgression is credited with restoring population numbers and decreasing genetic defects (Johnson et al., 2010; Saremi et al., 2019). This population is currently threatened by many factors such as habitat loss, intraspecific aggression, and disease (Johnson et al., 2010). Although several pathogens, e.g., feline leukemia virus and pseudorabies virus, have been reported to cause mortalities due to

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spill-over events from domestic cats or other hosts, some other parasites or pathogens may be host-specific (Glass et al., 1994; Miller et al., 2006; Yabsley et al., 2006; Cunningham et al., 2008, 2021; Shock et al., 2012; Elsmo et al., 2018; Chiu et al., 2019; Kraberger et al., 2020; Petch et al., 2022). Ecologically, when a species becomes extinct or is extirpated, host-specific parasites could also be lost (Perez and Palma, 2001; Davis et al., 2013; Malmberg et al., 2019).

Worldwide, wild and domestic felids are host to several *Babesia* spp. Several *Babesia* spp. have been reported from domestic cats from numerous countries and continents (Supplemental Table 1). In addition, uncharacterized species have been reported from domestic cats from Europe, Asia and Africa (Futter and Belonje, 1980; Stewart et al., 1980; Jittapalapong and Jansawan, 1993; Bourdeau, 1996; Moik and Gothe, 1997; Alvarado-Rybak et al., 2016). Immunocompromised and older cats may be more susceptible to infection, especially if they are coinfected with pathogens such as feline immunodeficiency virus (FIV) or canine distemper virus (CDV) (Barr et al., 1989; Munson et al., 2008). Clinical babesiosis in domestic cats is primarily associated with *B. felis*, but severe disease has recently been associated with *B. galileei* and *B. lengau* (Bosman et al., 2013, 2019; Baneth et al., 2024) In addition, clinical signs have also been documented in domestic cats infected with other *Babesia* species such as *B. canis*, *B. vogeli, B. presentii, B. gibsoni,* and *B. herpailuri* (Stewart et al., 1980; Baneth et al., 2004; Palmer et al., 2022; Remesar et al., 2022; Criado-Fornelio et al., 2003; Almendros et al., 2023). However, no clinical disease was noted in natural or experimental infection of domestic cats with some *Babesia* spp. such as *B. vogeli, B. hongkongensis*, *B. cati* and *B. leo* (Futter and Belonje, 1980; Lopez-Rebollar et al., 1999; Ayoob et al., 2010; de Oliveira et al., 2022; André et al., 2014, 2015, 2022; Almendros et al., 2024).

In contrast to domestic cats, wild felids rarely develop clinical disease due to *Babesia* infection; however, mortality due to babesiosis has been reported in African lions (*Panthera leo)* experiencing a CDV outbreak and stress due to drought (Munson et al., 2008). *Babesia* species reported from wild felids include *B. lengau* from cheetah (*Acinonyx jubatus*) from South Africa (Bosman et al., 2010), *B. felis* from African wild cats (*Felis lybica*), caracals (*Caracal*), cheetahs, lions, and servals (*Leptailurus serval*) from Africa (Penzhorn et al., 2004; Bosman et al., 2007; Williams et al., 2014), *B. leo* from lions and leopards (*Panthera pardus)* from South Africa and Zambia (Penzhorn et al., 2001; Bosman et al., 2007; Williams et al., 2014), *B. pantherae* from the African leopard (Dennig and Brocklesby, 1972), and *B. herpailuri* from the jaguarundi (*Puma yagouaroundi*) from Central America (Dennig, 1967). Undescribed species have been reported from the West African civet cat (*Civettictis civetta*) (Wenyon and Hamerton, 1930), the Indian leopard (*Panthera pardus fusca*) (Shortt, 1940), pampas cats (*Leopardus colocola*) and genets (*Viverra genetta*) in Brazilian zoos (André et al., 2011), bobcats (*Lynx rufus*) (Shock et al., 2013), and the Florida puma (Yabsley et al., 2006).

Only a single report of *Babesia* infection of Florida pumas has been published, with 90% of 39 Florida pumas tested being positive (Yabsley et al., 2006). Genetically, the parasite was related to members of the *Babesia* sensu stricto clade; however, little is known about the basic biology of this parasite. In the current study, we synthesize previous data and provide additional data on the morphologic, serologic, and molecular characteristics of this *Babesia* sp. Collectively, these data indicate the parasite is novel and we provide a formal description.

2. Materials and methods

2.1. Morphologic characteristics

Blood smears from Florida pumas with PCR confirmed *Babesia* infections (FP222 and FP93) were examined to obtain morphologic characteristics. Slides were fixed in methanol for 3 min, stained with Giemsa, and examined with an Olympus CH30 light microscope (Olympus Optical Co., Japan) under oil immersion (1000x). The length and width of each parasite was measured with an ocular micrometer.

2.2. Hematological evaluation

Hematologic data were compiled for 25 *Babesia*-infected free-ranging Florida pumas and compared with data from previous studies on Florida pumas and pumas from other populations (Currier and Russell 1982; Hawkey and Hart 1986; Dunbar et al., 1997; Rotstein et al., 1999; Foster and Cunningham, 2009). The samples were collected from 16 males and nine females from 2000 to 2005 from several locations in Hendry and Collier Counties.

2.3. Molecular characterization

Genomic DNA was extracted from whole blood samples using a QIAGEN DNeasy Blood and Tissue Extraction Kit (Germantown, MD, USA) following manufacturer's directions. To genetically characterize the *Babesia* species, portions of the nearly complete 18S rRNA gene, ITS-1 region, ITS-2 region, and partial sequences of the 28S rRNA, cytochrome *c* oxidase I (*COI*), cytochrome *c* oxidase IIII (COX3), β-tubulin gene, and cytochrome *b* (cytb) genes were amplified using primers listed in Table 1. To investigate intraspecific variation, we amplified and sequenced the internal transcribed spacer (ITS)-1 rRNA region from 23 Florida pumas (Shock et al., 2011). Samples originated from numerous counties throughout the Florida puma range (i.e., Collier, Broward, Dade, and Hendry Counties).

Amplicons were detected in a 0.8% agarose gel stained with GelRed (Biotium, Fremont, CA, USA). Amplicons were extracted from the gel using a QIAGEN gel extraction kit per manufacturer's directions. Bi-directional Sanger sequencing was conducted by the Georgia Genomics facility (Athens, GA, USA) and Genewiz (South Plainfield, NJ, USA) and the sequences were edited and assembled using Geneious 10.2.6 (Biomatters Limited, Auckland, New Zealand). Related sequences were obtained from GenBank, and phylogenetic trees were constructed in Geneious using an approximately maximum-likelihood method with FastTree v2.1 with a generalized time-reversible (GTR) model. Unique sequences were submitted to GenBank.

2.4. Distribution and prevalence in pumas

To investigate the prevalence of *Babesia* in Florida pumas and the possible presence of *Babesia* in pumas outside of Florida, DNA extracted from blood or spleen samples from 162 *P. concolor* was tested using an ITS-1 PCR assay as previously described (Shock et al., 2011). Samples originated from 131 Florida pumas from southern Florida, a single puma from Georgia (n = 1, genetically confirmed to be a Florida puma), and other subspecies of *P. concolor* from Texas (n = 24) (Belden and McCown, 1996), Louisiana (n = 1), North Dakota (n = 5), British Columbia, Canada (n = 9), and Costa Rica (n = 2).

2.5. Serologic analysis

Blood samples collected from six *Babesia*-positive pumas were tested for antibodies reactive to *Theileria equi* (n = 2), *B. odocoilei* (n = 4), *B. canis* (n = 2), and *B. bovis* (n = 2) antigens in an immunofluorescent antibody (IFA) assay as previously described (Lockhart et al., 1996; Yabsley et al., 2003). Briefly, whole blood was serially diluted from 1:2 to 1:256 with phosphate-buffered saline and placed on prepared *T. equi*, *B. odocoilei*, *B. bovis,* and *B. canis* antigen slides (*B. odocoilei* slides provided by S. Telford and others were purchased from Fuller Laboratories, Fullerton, CA (*B. canis* and *T. equi* slides) and ProtaTek International St. Paul, MN (*B. bovis* slides)) and then washed with phosphate-buffered saline. Antibodies were then incubated with 1:50 FITC-labeled goat anti-cat immunoglobulin G conjugate (Southern BioTech, Birmingham, Alabama). Slides were examined under a BH2 confocal microscope (Olympus, Japan). Sera from lab raised domestic cats served as negative

Table 1
PCR primers used for the detection of different gene targets of *Babesia* from Florida pumas (*Puma concolor coryi*) from Florida, USA.

Gene	Primer set (bp)	Sequence (5′-3′)	Reference(s)
18S rRNA gene	1°: 5.1/B (~1700)	CCTGGTTGATCCTGCCAGTAGT/TGATCCTTCTGCAGGTTCACCTAC	Medlin et al. (1988); Yabsley et al. (2006)
	2°: BabF/BabR (~650)	GTGAAACTGCGAATGGCTCA/CCATGCTGAAGTATTCAAGAC	Inokuma et al. (2003)
28S rRNA gene	ITS-F/LSU300 (245)	GAGAAGTCGTAACAAGGTTTCCG/TWGCGCTTCAATCCC	Holman et al. (2003)
ITS-1 region	1°: 15C/13B	CGATCGAGTGATCCGGTGAATTA/GCTGCGTCCTTCATCGTTGTG	Shock et al. (2012)
	2°: 15D/13C (~600–700)	AAGGAAGGAGAAGTCGTAACAAGG/TTGTGTGAGCCAAGACATCCA	Shock et al. (2012)
ITS-2 region	FOR7/REV7 (~200–300)	AGCCAATTGCGATAAGCATT/TCACTCGCCGTTACTAGGAGA	Shock et al. (2012)
cytochrome *c* oxidase I (*COI*)	coxF/coxR (1,085)	GGAAGTGGWACWGGWTGGAC/TTCGGTATTGCATGCCTTG	Schreeg et al. (2016)
Cytochrome *c* oxidase III (COX3)	Cox3F/Cox3R (~730)	ACTGTCAGCTAAAACGTATC/ACAGGATTAGATACCCTGG	Schreeg et al. (2016)
Cytochrome *b* (cytb)	CytBF/CytBR (~1100)	TTAGTGAAGGAACTTGACAGGT/CGGTTAATCTTTCCTATTCCTTACG	Thomas et al. (2023)
β-tubulin gene	Babtub1/Babtub3 (~1200)	CAAATWGGYGCMAARTTYTGGGA/ TCGTCCATACCTTCWCCSGTRTACCAGTG	Zamoto et al. (2004)

controls but no positive control samples were available.

2.6. Evaluate possible vertical transmission

Whole blood was collected from 24 kittens, all less than four months of age, born to 11 *Babesia*-positive mothers (range of 1–4 kittens/litter). Kittens were sampled during routine den checks by Florida Fish and Wildlife Conservation Commission (FWC) personnel. DNA was extracted from 100 µl of whole blood samples using the DNAeasy extraction kit (Qiagen, Germantown, MD) following the manufacturer's protocol. DNA was tested for *Babesia* spp. using nested PCR targeting the ITS1 rRNA gene as described (Shock et al., 2011).

Ethical statement

Blood and sera samples were collected from Florida pumas as part of routine captures for physical exams and research conducted by FWC personnel. Samples from cougars outside of Florida were collected from clinical case (mortality) submissions or from depredation animals. The use of samples that had been previously collected and archived was reviewed and approved by the University of Georgia's IACUC (A2009 12–220, A2010 10–186, and A2013 07-003).

3. Results

3.1. Description of Babesia coryicola sp. nov. Shock, cunningham, and yabsley

Type host: Female Florida puma (*Puma concolor coryi*) FP222 (Mammalia:Felidae)

Type locality: Collier County, Florida.

Type material: Hapantotype. A thin-stained blood smear from Florida puma FP222 from Collier County, Florida was deposited in the U.S. National Parasite Collection (USNPC accession number).

Parahapantotype. A thin-stained blood smear from Florida puma FP93 was deposited in the U.S. National Parasite Collection (USNPC accession number xxxxx).

Vector: Currently unknown.

Representative sequences: Genbank DQ329139 and PP852299 (18S rRNA gene); KJ592628 (ITS1 region); KJ592629 (ITS2 region); DQ329139 (β-tubulin gene); PP855655 (COI gene); PP855654 (cytb gene); PP855655 (COX3).

Etymology: *Babesia coryicola* sp. nov. is named after the subspecies name of the only known host, the endangered Florida puma *concolor coryi*. When the Florida puma was initially described in 1896 by Charles B. Cory it was named *Felis floridana* and was later renamed *Felis coryi* because *F. floridana* was previously used for the bobcat (*Felis rufus)*. Following the taxonomic revision of pumas by Nelson and Goldman (1929), the Florida puma was reclassified as a subspecies. Name: coryi-refers to the subspecies of the Florida puma and -cola is inhabitant of.

ZooBank reference: urn:lsid:zoobank.org:pub:F7CD2D01-3B51-4AAC-A30B-6D2240A07C17.

Description: All parasites observed (n = 18) were ring stage trophozoites which were morphologically similar to small *Babesia* spp., including felid-associated species such as *B. leo* and *B. felis* (Fig. 1). Most trophozoites were compact ring forms with small amounts of cytoplasm and a single nucleus, but rare trophozoites with 2–4 nuclei were observed. The overall shape of most trophozoites were round, but rare oval or slightly amoeboid forms were observed. Trophozoites measured 0.85–1.3 µm (0.991 µm average) in length and 0.75–1.2 µm (0.941 µm average) in width with no tetrad (Maltese cross) shapes observed (Fig. 1).

3.2. Natural history studies on host(s) and locations

The parasite has been detected in blood samples collected from Florida pumas sampled from 1989 to 2013. A previous survey of Florida pumas reported a prevalence of 95% (37 of 39 pumas) (Yabsley et al., 2006). In the current study, 72% of 100 Florida pumas were PCR positive for *B. coryicola* sp. nov. To date, only Florida pumas in their southern Florida (Collier, Hendry, Broward, Monroe, and Dale Counties) range have been infected. Five cougars from Texas that were translocated to Florida were negative for *B. coryicola* sp. nov. before their release and two of these animals resampled after release remained negative (Rotstein et al., 1999; Yabsley et al., 2006). Additionally, 19 pumas from Texas, introduced into northern Florida in 1993 and removed in 1995 for a reintroduction feasibility study, were also blood-smear negative for piroplasms prior to their introduction to Florida (Belden and McCown, 1996; Rotstein et al., 1999). Our results confirm that these pumas were negative for piroplasms; by PCR however, some of the pumas did acquire *C. felis* infections while in Florida (Rotstein et al., 1999). All pumas tested from Georgia, Louisiana, North Dakota, Canada, and Costa Rica were negative for piroplasms.

3.3. Serologic characteristics

Samples from two *B. coryicola* sp. nov.-positive Florida puma samples displayed fluorescence at a 1:128 dilution for *B. bovis* and *B. canis* antigens. Samples from four *B. coryicola* sp. nov. positive Florida pumas displayed fluorescence at 1:256 dilution for *B. odocoilei* antigens. No sample displayed cross-reaction for *T. equi*.

3.4. Molecular analysis

3.4.1. Near full-length 18S rRNA gene

The near full-length 18S rRNA gene sequences of *B. coryicola* sp. nov. were obtained from three Florida pumas (FP40, FP44, FP222). Sequences from FP40 and FP44 were identical and were 99.9% similar to FL222 (1608/1,610bp). These sequences were most similar to a *Babesia*

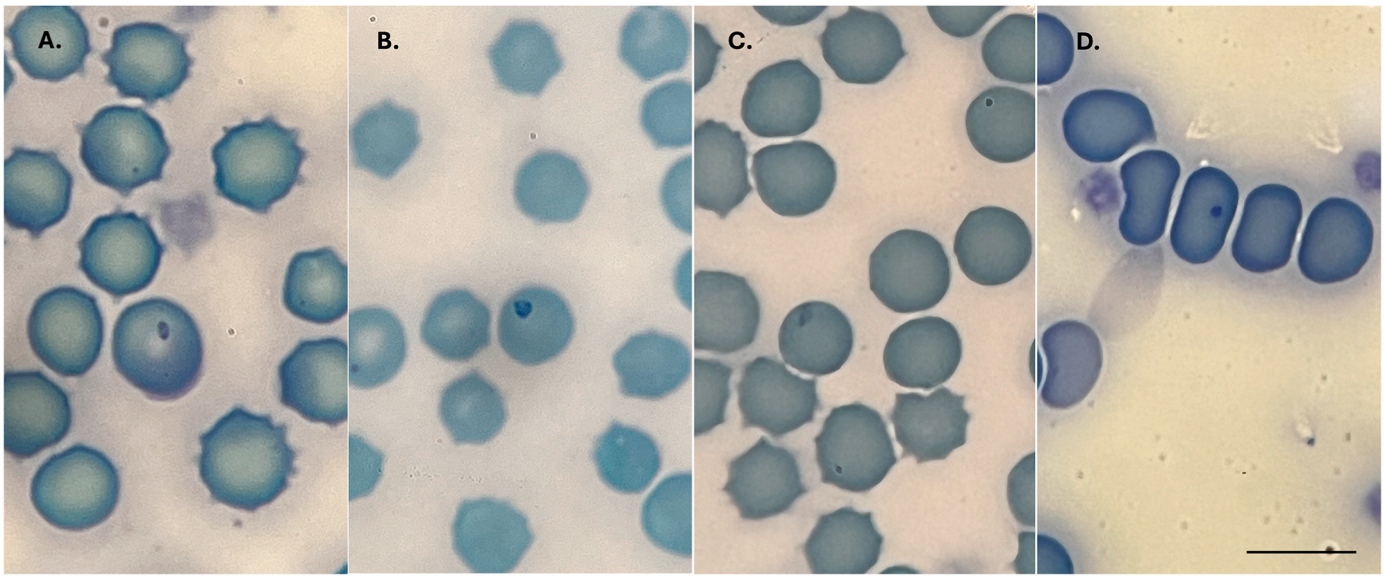

Fig. 1. Photomicrographs of *Babesia coryicola* sp. nov., type-material in blood smears from FP222 Florida puma (*Puma concolor coryi*) (A–C) showing ring and amoeboid trophozoites and FP93 (D) showing a compact ring form.

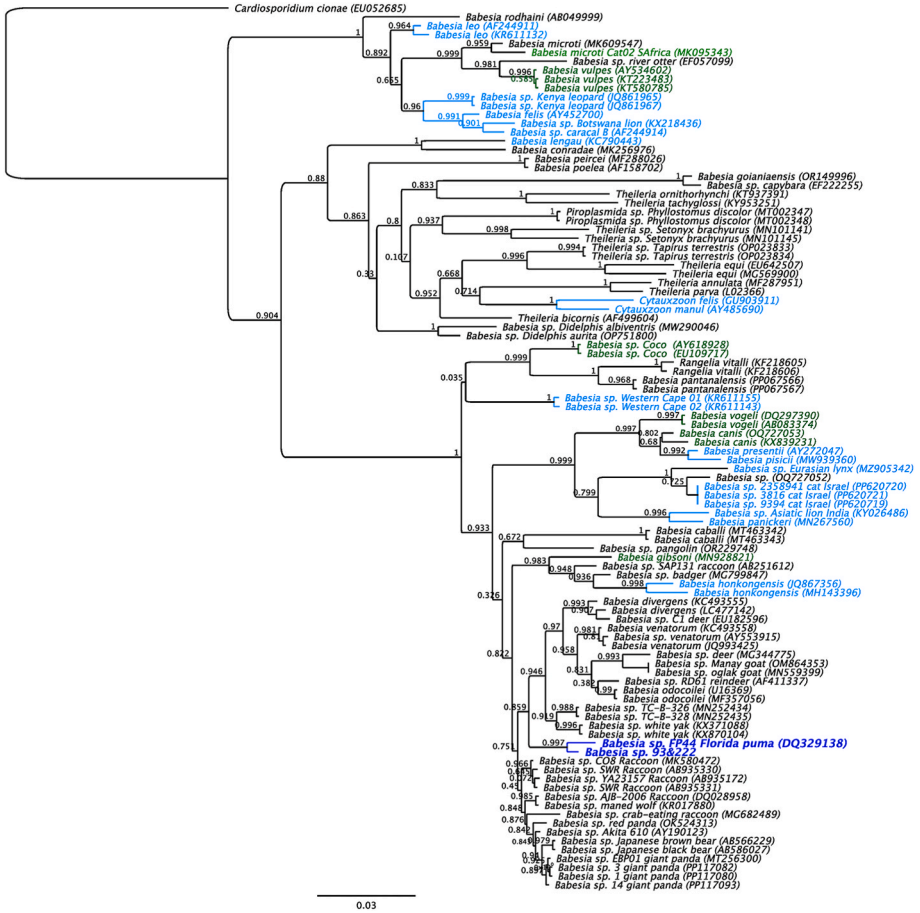

Fig. 2. Genetic relationships of *Babesia coryicola* sp. nov. from Florida pumas (*Puma concolor coryi*) compared with other *Babesia* spp. based on near full length 18S rRNA gene sequences. The text in bold blue in the figure represents specimens analyzed in this study. Sequences in light blue are species that have been primarily associated with felid hosts. Green lineages are predominately associated with canid hosts but have been reported in felids. Several sequences derived from domestic cats (i.e., MW578972, PP151898, and PP151899) and wild felids (i.e., HQ187782 and HQ187782) were not included in the analysis because the sequences were short.

sp. reported from a tick removed from a dog in Japan (Akita610) (98.3% (1651/1679), AY191024) and a *Babesia* sp. from a red panda from China (98.1% (1691/1724), OK524313) (Supplementary File 1 shows distance matrix with other *Babesia* spp.). Phylogenetically, *B. coryicola* sp. nov. grouped with other *Babesia* sensu stricto species as an ancestral group within a large group of *Babesia* spp. of ungulates (Fig. 2). This large group was a sister group to a clade of carnivore-infecting *Babesia* spp.

3.4.2. Partial β-tubulin gene

Partial β-tubulin gene sequences (1,101bp) of *B. coryicola* sp. nov. were obtained from three Florida pumas (FP44, FP93, and FP222). The β-tubulin gene sequences from *B. coryicola* sp. nov. from the Florida pumas were 99.5–99.7% similar to each other. These sequences were 87.3% similar to *B. odocoilei* (of 938-954bp overlapping with AY144706) and 87.2% similar to a *Babesia* sp. from a Sika deer (*Cervus nippon*) (of 940-956bp overlapping with KC465971) (Supplementary File 2 shows distance matrix with other *Babesia* spp.). Phylogenetically, the three *B. coryicola* sequences grouped together in a sister clade to *Babesia* spp. that primarily infect ungulates (Fig. 3).

3.4.3. Partial cytb gene

Partial cytb gene sequences (1,213bp) of *B. coryicola* sp. nov. were obtained from two Florida pumas (FP93 and FP222) and they were 99.8% similar to each other. The cytb gene sequences from *B. coryicola* sp. nov. from the Florida pumas were 84.6–84.8% similar to *B. canis* (KC207822) and 84.4% similar to a *B. vogeli* (KC207825) (Supplementary File 3 shows distance matrix with other *Babesia* spp.). Phylogenetically, the two *B. coryicola* sequences grouped as a sister group to a group of *Babesia* spp. of ungulates and carnivores (Fig. 4).

3.4.4. Partial COI gene

Partial COI gene sequences (1,035bp) of *B. coryicola* sp. nov. were obtained from two Florida pumas (FP93 and FP222) and they were 99.7% similar to each other. The COI gene sequences from *B. coryicola* sp. nov. from the Florida pumas were 89.2% similar to a *B.* sp. from a raccoon *(Procyon lotor)* (KR017882), 88.8% similar to a *B.* sp. from a captive maned wolf (*Chrysocyon brachyurus*) (KR017881), and 86.2% similar to *B. gibsoni* from a domestic dog in Japan (AB685183) (Supplementary File 4 shows distance matrix with other *Babesia* spp.). Phylogenetically, the two *B. coryicola* sequences grouped with a clade of *Babesia* sp. from raccoons (Fig. 5).

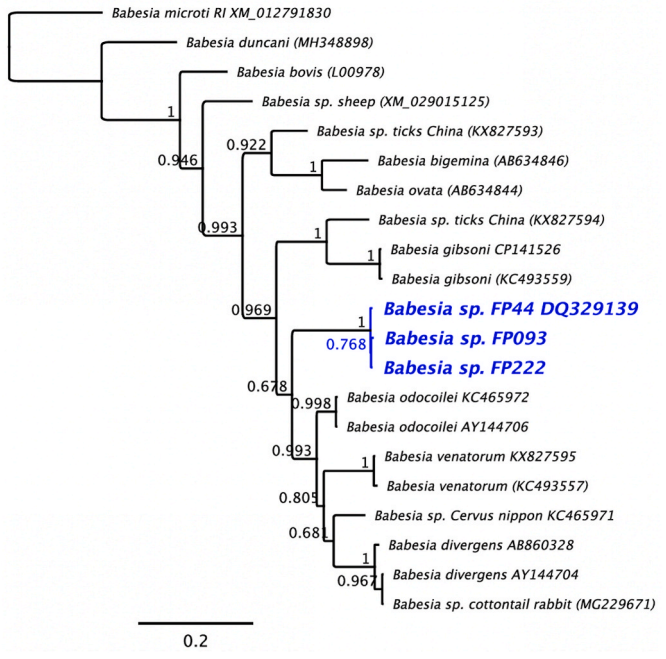

Fig. 3. Genetic relationships of *Babesia coryicola* sp. nov. from Florida pumas (*Puma concolor coryi*) compared with other *Babesia* spp. based on partial β-tubulin gene sequences. The text in bold in the figure represents specimens analyzed in this study.

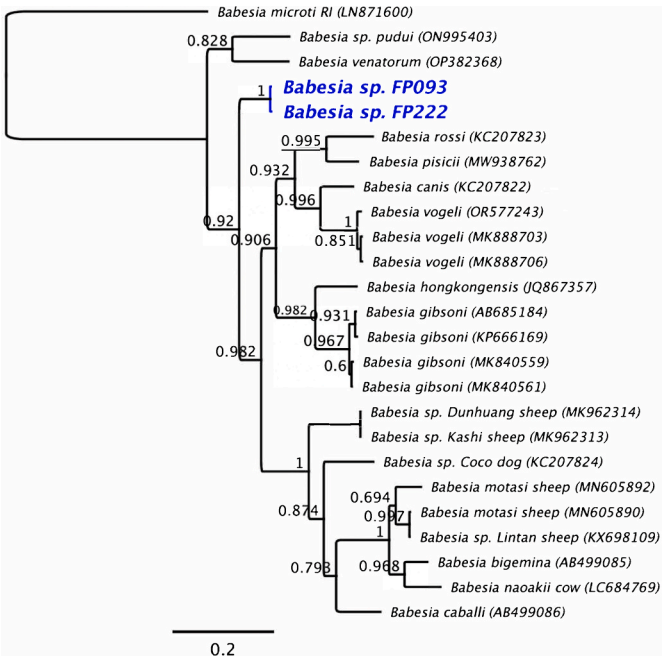

Fig. 4. Genetic relationships of *Babesia coryicola* sp. nov. from Florida pumas (*Puma concolor coryi*) compared with other *Babesia* spp. based on partial cytb gene sequences. The text in bold in the figure represents specimens analyzed in this study.

3.4.5. Partial COX3 gene

Partial COX3 gene sequences (717bp) of *B. coryicola* sp. nov. were obtained from four Florida pumas (FP93, FP222, FP233, and FP235). Each sequence was unique and were 99.4–99.9% similar to each other. The COX3 gene sequences from *B. coryicola* sp. nov. from the Florida pumas were most similar to *B. canis* (83.9–84.4% similar) followed by several strains of *B. gibsoni* (~81–82.8% similar) (Supplementary File 5 shows distance matrix with other *Babesia* spp.). Phylogenetically, the two *B. coryicola* sequences grouped with a clade of *B. gibsoni* sequences and this group was a sister clade to *B. canis* and *B. vogeli* (Fig. 6).

3.4.6. Partial 28S rRNA gene

A short region of the 28S rRNA gene (245bp) was obtained for *B. coryicola* sp. nov. from one Florida pumas (FP93). The sequence was most similar (95.5%) to a *Babesia* sp. from a red panda (OK524314) followed by *B. bigemina* (90.3%, LK391709) and *B. gibsoni* (CP141527).

3.4.7. ITS-1 and ITS-2 regions

The ITS-1 rRNA sequences of *B. coryicola* sp. nov. from 23 Florida pumas were all unique and percent identity ranged from 85.7 to 99.5%. The sequences varied in size (641-657bp) due to numerous 1-15bp insertions/deletions. No apparent association was noted between ITS-1 sequences and county of origin or year of sampling (range from 1992 to 2005). The *B. coryicola* sp. nov. ITS-1 sequences were 87.2–89.4% and 86.8–87.9% identical to *B. odocoilei* (AY339751) and *B. divergens* (EF458168), respectively. A representative sequence was submitted to GenBank (KJ592628, FP40).

The ITS-2 rRNA sequence of *B. coryicola* sp. nov. was obtained from one Florida puma (KJ592629; FP40). It was 244 bp long and contained a single polymorphic base. The ITS-2 sequence was 96% similar to

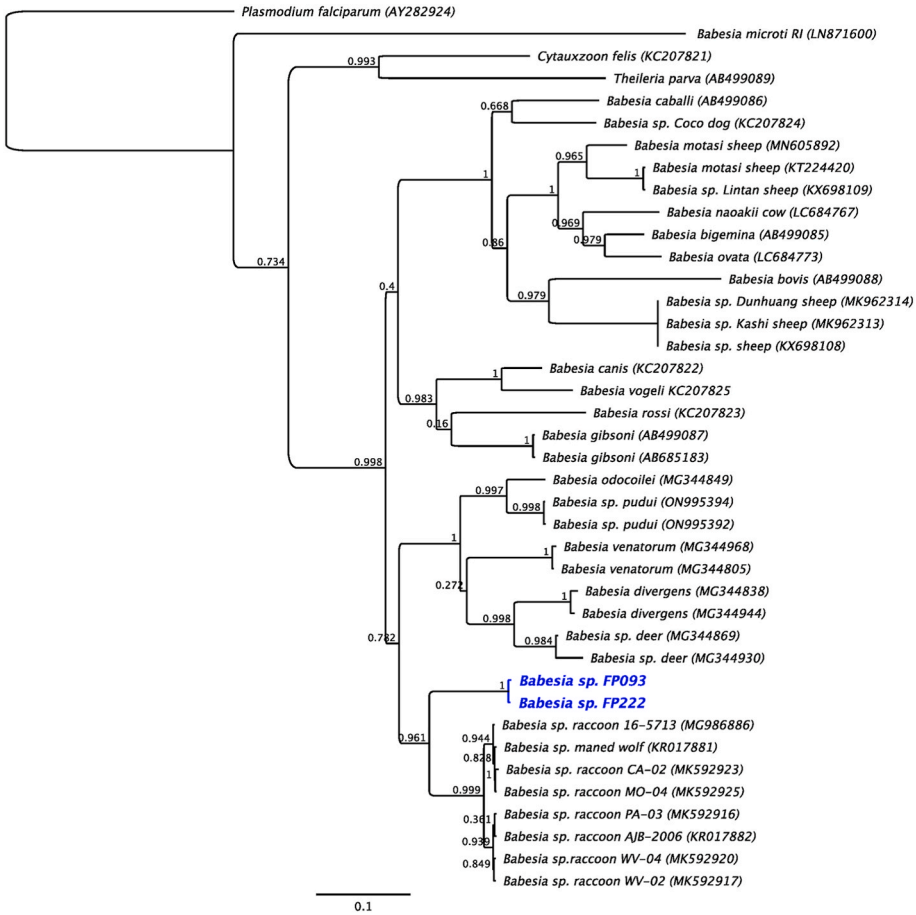

Fig. 5. Genetic relationships of *Babesia coryicola* sp. nov. from Florida pumas (*Puma concolor coryi*) compared with other *Babesia* spp. based on partial COI gene sequences. The text in bold in the figure represents specimens analyzed in this study.

B. bigemina (e.g., MH050926), 92% similar to a *B.* sp. from a red panda (OK524314) and *Babesia* sp. RD61 from a captive reindeer (*Rangifer tarandus*) from California (AY339744), and 91% similar to *B. odocoilei* from a Minnesota caribou *(Rangifer tarandus)* (AY339758).

3.5. Assessment of vertical transmission

All 24 kittens were PCR negative for piroplasms. During the year of sampling, all mothers were confirmed positive for *B. coryicola* sp. nov. by PCR.

3.6. Hematological evaluation

No hematological differences were detected between *Babesia*-infected Florida pumas included in this study and the seven groups of pumas from the five other studies (Table 2).

4. Discussion

Historically, the Florida puma was considered a reservoir host for *C. felis* based on the iatrogenic transmission of the parasite from a Florida puma in 1989 to a domestic cat (Butt et al., 1991). Subsequent examination of blood smears detected a high prevalence of piroplasms which were initially all believed to be *C. felis* based on similar morphology (Rotstein et al., 1999). However, a molecular-based study revealed that the majority of Florida pumas were infected with a *Babesia* sp., while only a small percentage were infected with *C. felis* (Yabsley et al., 2006). Similar to a previous survey (Yabsley et al., 2006), our data indicate that a high percentage of pumas are infected with this novel *Babesia* species. Sequence analysis of several gene targets confirm that this parasite represents a novel species.

In general, small piroplasms are morphologically similar; therefore, the use of molecular characterization has become increasingly important in the differentiation of this group of parasites. *Babesia coryicola* sp. nov. is morphologically indistinguishable from *C. felis* and several small felid *Babesia* spp. (e.g., *B. leo*, *B. felis*, and most stages of *B. lengau*), but it is genetically unique (Davis, 1929; Penzhorn et al., 2001; Yabsley et al., 2006; Bosman et al., 2010; McDermid et al., 2017; Meredith et al., 2019). Similarly to other studies, we noted variable serologic cross-activity between *B. coryicola* sp. nov. and antigens of several piroplasm species (Herwaldt et al., 1996, 2003; Lopez-Rebollar et al., 1999; Prince et al., 2010). The highest titers of reaction were noted for *B. odocoilei* antigens which suggests a closer relationship between *B. coryicola* sp. nov. and *B. odocoilei* compared to other species tested for serologic cross-reactivity in the current study. However, the serologic assay we used (IFA) has not been validated for use with Florida pumas and cross-reactivity among piroplasm species is poorly studied. Florida pumas naturally infected with *B. coryicola* sp. nov. had low parasitemias (<1%) in this study and in a previous study (Rotstein et al., 1999) which suggests that this is the natural host for this parasite. Similarly, cheetahs and lions naturally infected with *B. lengau* and *B. leo*, respectively, have asymptomatic infections with low parasitemias which can easily be missed if blood-smear analysis alone is used for diagnosis (Bosman et al., 2010).

For those pumas captured, all *B. coryicola* sp. nov.-infected Florida pumas appeared healthy and exhibited normal behavior prior to capture. In general, hematological values of *B. coryicola* sp. nov.-infected Florida pumas were within putative normal ranges of wild and captive

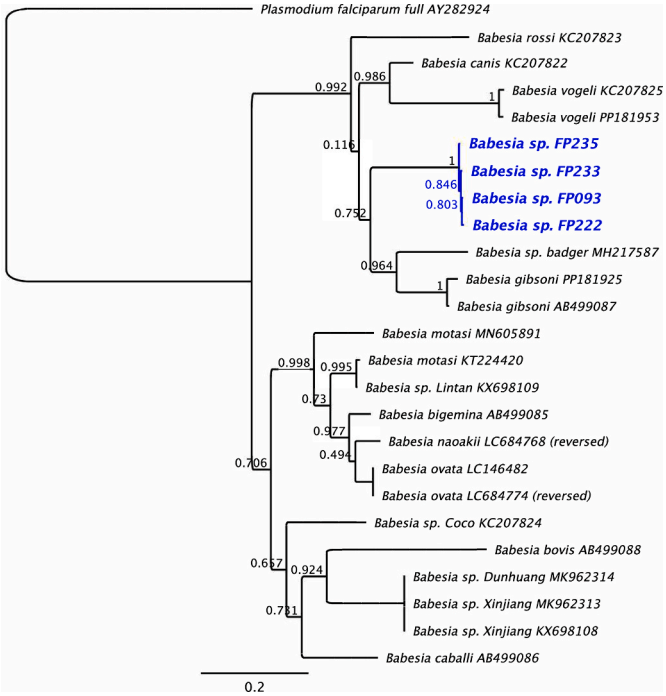

Fig. 6. Genetic relationships of *Babesia coryicola* sp. nov. from Florida pumas (*Puma concolor coryi*) compared with other *Babesia* spp. based on partial COX3 gene sequences. The text in bold in the figure represents specimens analyzed in this study.

P. concolor (Currier and Russell, 1982; Hawkey and Hart, 1986; Pospíšil et al., 1987; Paul-Murphy et al., 1994; Dunbar et al., 1997; Rotstein et al., 1999; Foster and Cunningham, 2009). Because anemia is a common clinical abnormality of clinical babesiosis (Schoeman et al., 2001), particular attention was given to various erythrocyte values (e.g., mean corpuscular hemoglobin, packed cell volume and erythrocyte counts), and although variability in data collection and analysis between studies precluded statistical analysis, no consistent differences were noted. Similarly, Rotstein et al. (1999), found no differences between several hematologic values between Florida pumas positive for piroplasms based on blood smear and Florida pumas that were blood-smear negative for piroplasms.

Phylogenetically, *B. coryicola* sp. nov. was consistently included as a member of the *Babesia* sensu stricto group; however, the exact placement varied by gene target analyzed which likely is related to the limited number of non-18S rRNA gene sequences available in Genbank. All analyzed targets confirmed that this parasite is novel and was not phylogenetically related to any *Babesia* spp. known to infect felids. Based on the 18S rRNA gene sequences, *B. coryicola* sp. nov. was included in a large clade of ungulate-infecting *Babesia* spp. This group was a sister group to a clade of *Babesia* species from carnivores including domestic dogs, domestic cats, lions, Eurasian lynx (*Lynx*), raccoons, and badgers. Phylogenetic analysis of the β-tubulin and cytb genes support the 18S rRNA gene results. The sequences of the ITS-1 and ITS-2 regions also suggest a close relationship with ungulate *Babesia* spp. However, the COI gene analysis suggested a closer relationship with *Babesia* sp. from raccoons, and the COX3 gene analysis suggested a closer relationship with *B. gibsoni*. The high prevalence of *B. coryicola* sp. nov. in Florida pumas is similar to data from raccoons and North American river otters (*Lontra canadensis*) which both have a high prevalence of *Babesia* (often prevalence >90%) (Birkenheuer et al., 2006, 2007; Garrett et al. 2019, 2022). The amount of intraspecific variability among the ITS-1 sequences was similar to data from other piroplasms (e.g., Holman et al., 2003; Aktas et al., 2007).

Ixodid ticks serve as the vector for all mammalian *Babesia* for which the life cycle has been determined. However, a tick vector has not been identified for any of the primarily feline infecting *Babesia* species nor the *Babesia* from raccoons (Birkenheuer et al., 2006, 2007; Garrett et al., 2019). To date, six tick species have been reported from the Florida puma including *Dermacentor variabilis*, *Dermacentor nitens*, *Ixodes scapularis*, *Ixodes keiransi* (=*I. affinis*), *Amblyomma americanum*, and *Amblyomma maculatum* (Forrester et al., 1985; Forrester, 1992; Wehinger et al., 1995; Hertz et al., 2017). Two of these tick species are unlikely vectors because *A. americanum* is rarely found on Florida pumas in their native range and has been primarily reported from captive pumas in northern Florida, and *D. nitens* has not been found on Florida pumas surveyed from 1989 to 2014 (Wehinger et al., 1995; Yabsley unpublished). In previous studies, *D. variabilis* and *I. scapularis* were the two most common ticks detected on pumas (Forrester et al., 1985; Forrester, 1992; Wehinger et al., 1995; Hertz et al., 2017). Additional studies are needed to identify which tick species, if any, are involved in transmission of *B. coryicola* sp. nov. because both *D. variabilis* and *I. scapularis* were commonly detected on our infected pumas (Yabsley unpublished data).

Although tick-borne transmission is the predominant method for *Babesia* spp. transmission, alternative transmission routes have been confirmed or are suspected for several *Babesia* spp. (e.g., blood transfusion, vertical, or intraspecific aggression) (Stegeman et al., 2003; Fukumoto et al., 2005; Johnson et al., 2009; Georges et al., 2011). In the current study, we found no evidence of vertical transmission; however, this route has been confirmed for several piroplasm species including *B. gibsoni* in dogs (Fukumoto et al., 2005), *B. microti* in humans (Fox et al., 2006), a *B. microti*-like sp. in baboons (*Papio cynocephalus*) (Bronsdon et al., 1999), and *T. equi* in horses (Phipps and Otter, 2004; Allsopp et al., 2007; Georges et al., 2011). A previous study identified piroplasms in the blood smear of a 7-day-old Florida puma kitten, but unfortunately, the piroplasm was not identified using molecular techniques (Rotstein et al., 1999). This piroplasm in the kitten could have been *C. felis,* but a limited study of *C. felis* in two pregnant domestic cats failed to document vertical transmission (Lewis et al., 2012); however, vertical transmission has been documented in various *Babesia* and *Theileria* spp. (Nietfeld and Pollock, 2002; Baek et al., 2003; Georges et al., 2011; Mierzejewska et al., 2014; Tołkacz et al., 2017). Fighting has been suggested as an alternative route of transmission for *B. gibsoni* among traditional fighting dog breeds including pit-bull-type dogs in the United States and Tosa dogs in Japan (Birkenheuer et al., 2005; Miyama et al., 2005; Yeagley et al., 2009). One of the major causes of mortality (40.4%) among radio-collared Florida pumas is intraspecific aggression (Taylor et al., 2002), but it is unknown if *B. coryicola* sp. nov. can be transmitted during fighting.

The historical distribution of *Babesia coryicola* sp. nov. is unknown, but testing of pumas from several populations in North and Central America was uniformly negative for *B. coryicola* sp. nov. Historically, *Puma concolor* was widespread throughout North, Central, and South America, but the eastern population in North America (*Puma concolor couguar*) was extirpated, except for an isolated population in southern Florida that is arguably classified as a separate subspecies by some researchers and regulatory agencies (Culver et al., 2000) and the US Fish and Wildlife Service. In addition to our data on negative pumas, two previous studies of Brazilian free-ranging and captive wild felids failed to detect *Babesia* spp.in pumas (*P. c. concolor*); although *Cytauxzoon* was detected from two captive and two free-ranging pumas (André et al., 2009; Silva et al., 2021). An additional study of Brazilian captive and wild felids found antibodies to *B. canis* in 11% of 18 pumas, but *Babesia* DNA was not detected in pumas (André et al., 2011). During a study of piroplasms in bobcats, only one road killed bobcat of 799 sampled from throughout the eastern US were positive for a *Babesia* sp. using the ITS-1 gene target, and it was not genetically related to *B. coryicola* sp. nov.; however, bobcats sampled in Florida were from northern counties (Shock et al., 2013). In the United States, no *Babesia* infections have been reported from domestic cats, but surveillance is limited.

Table 2
Hematological values for 25 *Babesia coryicola* sp. nov.-infected Florida pumas (*Puma concolor coryi*) compared to hematological values for *Puma concolor* from previous studies.

	Florida (n = 25)	Florida (n = 63)	Texas (n = 28)	Florida (n = 32)	Florida (n = 48)	Colorado (n = 43)	Colorado (n = 22)	London zoo (n = 29)	Brazil zoo (n = 4)	Brazil wildlife center (n = 5)
Species	*P. c. coryi*	*P. c. coryi*	*P. c. cougar*	*P. c. coryi*	*P. c. coryi*	*P. c. cougar*	*P. c. cougar*	*P. concolor*	*P. concolor*	*P. concolor*
Parameters:	Mean (Range)	Mean (SD)	Mean (SD)	Mean (Range)	Mean (SD)	Mean (95% CI)	Mean (95% CI)	Mean (Range)	Range	Mean (SD)
Age (years)	7.2 (0.6–13.3)	5 (0.02–8)[a]	5 (0.02–8)[a]	(3–10)[a]	(0.5–13.0)[a]	non-kitten, 1+	non-kitten, 1+	NR	2–18	NR
MC Hb (mg/dl)	10.9 (7.2–14.8)	11.9 (1.7)	12.8 (2.5)	10.8 (3.6–14.8)	12.21 (1.70)	17.8 (15.6–20.0)	NR	13.8 (10.5–17.8)	9.7–14.8	12.96 ± 1.92
HCT/PCV (%)	33.7 (22.5–45.7)	37.8 (5.7)	38.2 (8.3)	34.25 (12.9–47.7)	36.37 (5.30)	41.8 (38.9–43.7)	46.9 (44.9–48.8)	38.0 (27.0–48.0)	44–46.7	37.64 ± 5.16
WBC ($x10^3$/μL)	9.5 (4.8–16.6)	11.5 (3.7)	7.8 (3.6)	9.31 (3.9–37.5)	12.2 (3.0)	9.6 (4.2–15)	NR	6.4 (4.1–8.2)	5.2–8.5	6.94 ± 1.44
RBC ($x10^6$/μL)	7.2 (4.2–10.6)	7.8 (1.3)	7.9 (1.6)	7.11 (2.72–10.6)	7.635 (1.033)	10.25 (9.04–11.46)	NR	7.9 (6.0–9.7)	6.7–10.3	9.25 ± 1.16
MCV (fl)	47.2 (43–54)	48.6 (6.3)	48.7 (5.4)	48.3 (43.0–54.0)	47.29 (2.89)	28.76 (25.51–32.01)	NR	49 (46–54)	44–46.7	40.68 ± 2.36
MCH (pg)	15.3 (14–17.4)	15.3 (2.7)	16.2 (0.9)	15.26 (13.2–17.0)	16.07 (1.41)	NR	NR	17.6 (15.6–19.0)	14.1–15.7	15.9 ± 1.0
MCHC (g/dl)	32.4 (29.8–36.6)	31.1 (3.5)	33.6 (2.3)	31.62 (27.9–34.4)	34.08 (3.26)	NR	NR	35.8 (32.0–38.2)	31.8–33.7	34.4 ± 1.15
Platelets ($x10^3$/μL)	324.1 (139–642)	NR	NR	324.7 (99–642)	402.6 (131.5)	NR	NR	203 (145–280)	117–438	285.60 ± 94.13
Neutrophils ($x10^3$/μL)	6.8 (3.4–14	7.7 (3.1)	4.8 (3.1)	7.1 (2.9–29.3)	8.0 (2.9)	NR	NR	4.5 (3.2–6.4)	3.5–5	4.04 ± 1.16
Lymphocytes ($x10^3$/μL)	1.7 (0.5–2.6)	2.8 (1.5)	2.4 (1.7)	1.4 (0.4–6.4)	3.4 (1.7)	NR	NR	1.7 (0.8–2.5)	1.8–8.8	2.6 ± 0.56
Monocytes (cells/μL)	429.2 (121–1020)	563 (299)	298 (299)	300.5 (42–744)	390 (340)	NR	NR	100 (0–300)	126–280	150 ± 65
Eosinophils (cells/μL)	322.2 (0–774)	482 (227)	242 (220)	462.2 (0–1570)	420 (310)	NR	NR	100 (0–400)	0	90 ± 103
Basophils (cells/μL)	10.5 (0–124)	58 (49)	73 (111)	5.4(0–124)	100 (60)	NR	NR	NR	0	29 ± 42
Neutrophils (%)	72.8 (50–88)	NR	NR	76.4(61–89)	64.3 (14.3)	60.7 (58.5–52.9)	60.7 (58.5–52.9)	71 (47–85)	NR	NR
Lymphocytes (%)	18.6 (5.0–26)	NR	NR	14.6(7–36)	28.8 (14.5)	35.1 (32.7–37.5)	35.1 (32.7–37.5)	25 (14–36)	NR	NR
Monocytes (%)	4.7 (1.0–12)	NR	NR	3.3(1–6)	3.2 (2.6)	1.9 (1.6–2.2)	1.9 (1.6–2.2)	(0–5)	NR	NR
Eosinophils (%)	3.6 (0–10.0)	NR	NR	5.5 (0–12)	3.4 (2.2)	2.3 (1.9–2.7)	2.3 (1.9–2.7)	(0–6)	NR	NR
Basophils (%)	0.1 (0–1.0)	NR	NR	0.1 (0–1)	0.89 (0.57)	NR	NR	0	NR	NR
Status	free-ranging	free-ranging	captive	free-ranging	free-ranging	captive	free-ranging	captive	captive	Captive, under rehabilitation
Reference	Current study	Rotstein et al. (1999)	Rotstein et al. (1999)	Foster and Cunningham (2009)	Dunbar et al. (1997)	Currier and Russell (1982)	Currier and Russell (1982)	Hawkey and Hart (1986)	(Dias et al., 2022)	Silva et al., 2020,[b]

WBC = white blood cells, RBC = red blood cells, MC = mean corpuscular, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin. MCHC = mean corpuscular hemoglobin concentration.

[a] Range, SD = standard deviation, CI = confidence interval, NR = not reported, HCT/PCV = Hematocrit/Packed Cell Volume.

[b] Data presented are pumas with no detectable piroplasm infections. Data on an additional six animals that were infected with *Cytauxzoon felis* were also available in the study. The only significant differences between the two groups were that infected pumas had a decreased number of lymphocytes.

In summary, we describe *B. coryicola* sp. nov as a new species of *Babesia* that infects the Florida puma as a natural host. This parasite was found at a high prevalence in sampled pumas and was detected in pumas sampled from as early as 1989. Although we present data on the morphologic, molecular, and serologic characteristics of this new species, there are many questions that remain regarding its natural history. For example, the susceptibility of other felids, including domestic cats, is unknown. Additionally, similar to other felid *Babesia*, the vector has not been determined nor have other alternative routes of transmission been confirmed. Worldwide, this description adds to the growing list of *Babesia* spp. described from wild felids and is only the second felid *Babesia* described from the New World. Because numerous uncharacterized piroplasms have been reported from felids, additional studies are needed to fully understand the diversity and ecology of felid *Babesia* species.

CRediT authorship contribution statement

Barbara C. Shock: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Håkon H. Jones:** Writing – review & editing, Writing – original draft, Formal analysis. **Kayla B. Garrett:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Sonia M. Hernandez:** Writing – review & editing, Writing – original draft, Resources. **Holly J. Burchfield:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Katie Haman:** Writing – review & editing, Resources. **Helen Schwantje:** Writing – review & editing, Writing – original draft, Resources. **Sam R. Telford:** Writing – review & editing, Writing – original draft, Resources, Methodology. **Mark W. Cunningham:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Michael J. Yabsley:** Writing – review & editing, Writing –

original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property. We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from myabsley@uga.edu.

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Appendix A. Supplementary data

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