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## Molecular Detection and Characterization of *Cytauxzoon felis* and a *Babesia* Species in Cougars from Florida

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ABSTRACT: Piroplasms, morphologically indistinguishable from *Cytauxzoon felis*, previously were detected in 36% of cougars in Florida. We utilized a nested 18S rRNA assay, which amplifies DNA from all piroplasms, to screen blood samples collected from 41 cougars from Florida (39 native Florida panthers [*Puma concolor coryi*] and two translocated Texas cougars [*P. c. stanleyana*]) from 1989–2005. Thirty-nine of the 41 cougars (95%) were positive for piroplasms; however, sequence analysis and restriction enzyme digestion revealed that only five were positive for *C. felis*. Samples from 32 cougars were positive for a *Babesia* sp. Two cougars were co-infected with both *C. felis* and the *Babesia* sp. Phylogenetic analysis of 18S rRNA gene sequence indicated that the Florida panther *Babesia* sp. was most closely related to a *Babesia* sp. reported from *Ixodes ovatus* from Japan, *Babesia divergens*, and *Babesia odocoilei*. This study indicates that Florida panthers harbor two distinct piroplasms, *C. felis* and a *Babesia* sp., and that some individuals are infected with both organisms. The infectivity and pathogenicity of this *Babesia* sp. for domestic cats is unknown. This represents the first report of a feline *Babesia* sp. in North America.

*Key words:* *Babesia*, cougar, *Cytauxzoon*, feline, Florida panther, molecular analysis, piroplasm, phylogenetic analysis, *Puma*.

Multiple piroplasms have been reported from domestic and wild felids. Piroplasms reported from domestic cats include *Babesia felis* from Africa (Penzhorn et al., 2004), *Babesia cati* from India (Mudaliar et al., 1950), *Babesia canis canis* from Spain and Portugal (Criado-Fornelio et al., 2003), *Babesia microti*-like species (previously referred to as *Theileria annae*) from Portugal (Criado-Fornelio et al., 2003), *B. canis presentii* from Israel (Baneth et al., 2004), *Cytauxzoon felis* from North America and Africa (Wagner, 1976; Allsopp et al., 1994), and *Cytauxzoon* sp. from Europe (Criado-Fornelio et al., 2004). Uncharacterized species have been reported from France, Germany, Thailand, and Zimbabwe (Stewart et al., 1980; Jittapalapong and Jansawan, 1993; Bourdeau, 1996; Moik and Gothe, 1997).

Species reported from wild felids include *B. felis* from African wild cat (*Felis silvestris*) and caracal (*Felis caracal*) from Africa (Penzhorn et al., 2004), *Babesia herpailuri* from the jaguarundi (*Herpailurus yaguarondi*) from Central America (Dennig, 1967), *Babesia pantherae* from the leopard (*Panthera pardus*) from Africa (Dennig and Brocklesby, 1972), *Babesia leo* from lions (*Panthera leo*) from South Africa (Penzhorn et al., 2001), *Cytauxzoon manul* from the Mongolian Pallas' cat (*Otocolobus manul*) (Reichard et al., 2005), and *C. felis* from bobcats (*Lynx rufus*) (Glenn et al., 1983) and Florida panthers (*Puma concolor coryi*) (Butt et al., 1999) from North America. Undescribed species have been reported from the West African civit cat (*Viverra civetta*) (Wenyon and Hamerton, 1930), the Indian leopard (*Panterha pardus fusca*) (Shortt, 1940), and the cheetah (*Acinonyx jubatus*) (Averbeck et al., 1990).

Morphologically, piroplasms are broadly classified as small piroplasms (<1.5 μm) or as large piroplasms (>2.5 μm). Small feline piroplasms include *B. felis, B. leo*, and *Cytauxzoon* species and large feline piroplasms include *B. cati, B. pantherae, B. canis canis, B. canis presentii*, and *B. herpailuri*. Based on molecular analyses,



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the piroplasms are classified into four major groups, 1) the true babesias, *Babesia* sensu stricto, 2) *Theileria* and *Cytauxzoon* species, 3) the western piroplasms from wildlife and humans, and 4) *B. microti* and related small babesias (Kjemtrup et al., 2000b). The two subspecies of *B. canis* reported from domestic cats are included within the true babesias (group 1) (Criado-Fornelio et al., 2003; Baneth et al., 2004), the *Cytauxzoon* species are included within the *Theileria/Cytauxzoon* complex (group 2) (Criado-Fornelio et al., 2004), and the African felid parasites (*B. leo* and *B. felis*) and Portuguese *B. microti*-like species (referred to as *T. annae*) are included within the *B. microti*-small babesias complex (group 4) (Penzhorn et al., 2001). The phylogenetic relationships of *B. pantherae* and *B. herpailuri* to other piroplasms are unknown.

*Cytauxzoon felis* was first detected in a naturally infected Florida panther when a domestic cat inoculated with a blood sample from a panther developed fatal cytauxzoonosis (Butt et al., 1991). In a later study, piroplasms morphologically consistent with *C. felis* were detected in blood smears from 35% of Florida panthers and 39% of translocated Texas cougars (*P. c. stanleyana*) (Rotstein et al., 1999). Infection of the Texas cougars was assumed to have occurred in quarantine in northern Florida because piroplasms were not observed in blood smears prior to shipment from Texas (Rotstein et al., 1999). The identification of these piroplasms as *C. felis* was based on their morphology and the prior finding that inoculation of a domestic cat with mononuclear cells from a Florida panther resulted in fatal cytauxzoonosis (Butt et al., 1991). No clinical cytauxzoonosis has been reported from cougars in Florida, but a fatal cytauxzoonosis has been reported in the natural reservoir, the bobcat (*Lynx rufus*) (Nietfeld and Pollock, 2002), and in a captive white tiger (*Panthera tigris*) from northern Florida, USA (Garner et al., 1996). Two cases of fatal babesiosis have been reported in cougars translocated from a zoological park in California, USA to Cairo, Egypt (Carpano, 1934). Both cougars were housed near other wild felids (lions, tigers, and leopards) and were infested by *Rhipicephalis simus*, a tick common in northern Africa. The cougars were believed to have been infected with the *Babesia* sp. (referred to as *Babesiella felis*) while in Africa (Carpano, 1934).

The objectives of the current study were to determine the prevalence of piroplasms in cougars from Florida using a polymerase chain reaction (PCR) assay and genetically characterize the small piroplasms of cougars by sequence analysis of the 18S rRNA and β-tubulin genes.

A total of 41 blood samples for this study were collected from Florida panthers and Texas cougars during routine biannual health screening and radio telemetry collaring. From 1989 to 1995, whole-blood samples were collected from nine Florida panthers and two Texas cougars and from 2003 to 2005, samples were collected from 30 Florida panthers. The Texas cougars were trapped in western Texas and translocated to the Florida Game and Fresh Water Fish Commission's Wildlife Research Laboratory in Gainesville, Florida (29°39′05″N, 82°19′30″W) where they were quarantined prior to release in southern Florida as part of a genetic restoration project for the Florida panther. All blood samples were collected in EDTA tubes and submitted for complete blood count (CBC), packed cell volume, hemoglobin concentration, and blood smear analysis for piroplasms, results of which have been reported (Rotstein et al., 1999). Remaining blood was frozen at −20 C until molecular analysis in the current study.

The DNA was extracted from 100 μl of whole-blood samples with the use of the GFX Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) in accordance

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with the manufacturer's protocol. For PCR analysis of the 18S rRNA gene, primary outside amplification was conducted with 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, USA), 2.5 units *Taq* DNA Polymerase (Promega), and 0.8 μM of primers A and B (Medlin et al., 1989). Cycling parameters for the primary amplification was 94 C for 1 min followed by 35 cycles of 94 C for 1 min, 48 C for 1 min, and 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, RLB-F and RLB-R (Gubbels et al., 1999), were used. Cycling conditions were the same as primary reaction except the annealing temperature was 50 C. To obtain full length 18S rRNA gene sequence, overlapping sequences were amplified using two PCR protocols. The 5′ end was obtained using primers 5.1 (Yabsley et al., 2005) and B (Medlin et al., 1989) in a primary reaction and primers 5.1 and 3.1 (Yabsley et al., 2005) in a secondary reaction, and the 3′ end of the 18S rRNA gene and the ITS-1 region was amplified using primers 15C (5′-CGATCGAGTGATCCGGTGAATTA) and 13B (5′-GCTGCGTCCTTCATCGTTGTG); PCR reaction conditions were the same as for primers A and B above, except that 52 C was used as the annealing temperature. Sequencing was conducted using primers 5.1, RLB-F, RLB-R, 3.1, 15C, and 13B. Partial β-tubulin gene sequences were amplified and sequenced from two *Babesia*-positive cougars using primers Tubu63F and Tubu-3′ as described (Zamoto et al., 2004).

Restriction analysis of 18S rDNA (PCR-RE) was done with the use of restriction enzyme *HpaII* as recommended by the manufacturer (Promega). Ten microliters of secondary 18S rRNA PCR products were digested with 2 μl of *HpaII* enzyme in a 20-μl reaction containing 2 μl of 10X buffer (Promega), 2 μl of a 1 : 10 dilution of BSA, and 4 μl of water. This reaction was incubated at 37 C for 2 hr, followed by an enzyme-inactivation step of 70 C for 15 min. Resulting restriction profiles were analyzed in 2.5% agarose gels, stained with ethidium bromide, and visualized with UV light.

Representative products were purified with a Microcon spin filter (Amicon Inc., Beverly, Massachusetts, USA), sequenced at MWG-BIOTECH (High Point, North Carolina, USA), and the resultant sequences compared to published sequences in the GenBank database. Stringent protocols and controls were utilized in all PCR assays to prevent and detect contamination. DNA extraction, primary amplification, secondary amplification, and product analysis were performed in separate dedicated laboratory areas. Two negative water controls were included in each set of DNA extractions, and one water control was included in each set of primary and secondary PCR reactions.

Resultant sequences were compared to sequences available in the GenBank database, except for the Portuguese *Babesia microti*-like species (referred to as *T. annae*; AY150068) because only 415 bases were available. The ClustalX program was used for sequence alignment followed by neighbor-joining algorithm phylogenetic analysis with the use of the Kimura 2-parameter model with the MEGA (Molecular Evolutionary Genetics Analysis) program. The MEGA program was also used to determine percent identity by pairwise comparison. The 18S rRNA and β-tubulin gene sequences of the *Babesia* sp. have been deposited in the GenBank database (Accession numbers DQ329138 and DQ329139, respectively).

With the use of the nested 18S rRNA gene assay, 39 of the 41 (95%) cougars tested were positive for piroplasms, of which 37 of 39 (94.9%) of the Florida panthers and both of the Texas cougars were positive. The suitability of *HpaII*

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TABLE 1. Results of PCR and restriction enzyme profile analyses for *Cytauxzoon felis* and *Babesia* sp. in Florida panthers and Texas cougars from Florida.

| | Number PCR positive for piroplasms (%) | Restriction enzyme profile identification Number positive (%) | | |
|---|---|---|---|---|
| | PCR | *C. felis* | *Babesia* sp. | *C. felis* and *Babesia* sp. |
| Florida panther ($n$=39) | 37 | 3 | 32 | 2 |
| Texas cougar ($n$=2) | 2 | 2 | 0 | 0 |
| Total | 39 | 5 | 32 | 2 |

digestion differentiation of *C. felis* from other piroplasms was based on sequence analysis of available sequences in Genbank. This analysis indicated that only the secondary 18S rRNA gene amplicon for *C. felis* contained a restriction site resulting in two fragments of 74 bp and 440 bp. Based on the PCR-RE assay, only five of the 39 PCR positive cougars produced the expected restriction profiles for *C. felis* (Table 1). Samples from 32 panthers were not cut by *HpaII* suggesting infection with a different piroplasm. The remaining two samples had two equal strength bands corresponding to both cut and uncut restriction profiles suggesting co-infection.

Three amplicons of each restriction profile were sequenced; two from Texas cougars and four from Florida panthers. The sequences from both Texas cougars and one Florida panther were 100% identical to each other and to the Texas, Oklahoma, and South Africa *C. felis* sequences in Genbank (AY531524, AF-399930, L19080) confirming the results of the PCR-RE analysis. Sequences from the other two Florida panthers were identical to each other and most closely matched members of the *Babesia* genus. Co-infection of the two panthers with both restriction enzyme profiles was confirmed by sequence analysis of the ITS-1 gene (data not shown).

The full-length 18S rRNA gene sequence (1,734 bases) of the *Babesia* sp. from the Florida panther shared the highest homology with a *Babesia* sp. Fukui766 (AY190124) detected in the tick *Ixodes ovatus* from Japan (Inokuma et al., 2003) (98.6% similarity). Alignment of the 1,678 bp of the 18S rRNA gene sequence of the panther *Babesia* sp. which overlapped with available sequences in GenBank of related piroplasms and *Plasmodium falciparum* (as outgroup, M19172) resulted in an alignment 2,029 bp in length, of which 1,071 were invariant, 277 variable characters were parsimony uninformative, and 371 were parsimony informative.

Both analyses (neighbor-joining and maximum parsimony) produced trees with the same major clades of piroplasms previously reported (Kjemtrup et al., 2000a, 2000b; Goethert and Telford, 2003). Both analyses placed the panther *Babesia* sp. in the *Babesia* sensu stricto clade as a single-member sister clade to the two Japanese *Babesia* sp. from in *I. ovatus* (Fig. 1); however, the maximum parsimony analysis was less robust in supporting this relationship (bootstrap = 56; data not shown). The percent identities of the 1,678 overlapping bp of the *Babesia* sp. from Florida panthers compared with selected piroplasms, including those reported from felids, are shown in Table 2.

Sequences of the β-tubulin gene (1,198 bp) for two *Babesia*-positive panthers were identical and most similar to partial sequences from *Babesia odocoilei* (87%; AY144705-6) and *Babesia divergens* (85%; AY144703-4). Alignment of 865 bp of the β-tubulin gene sequence of the panther *Babesia* sp., which overlapped with sequences available for related piroplasms and *Toxoplasma gondii* (as outgroup,

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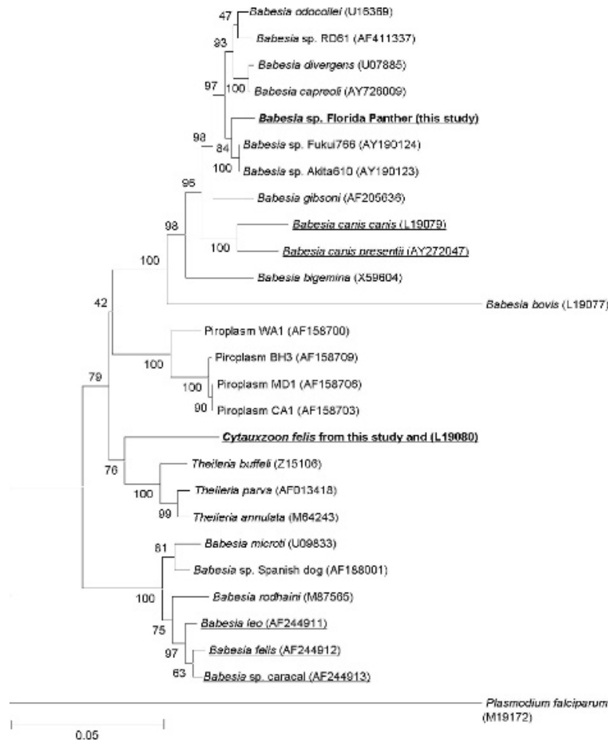


FIGURE 1. Phylogenetic tree inferred by comparisons of 18S rRNA gene sequences among *Babesia* spp., *Theileria* spp., *Cytauxzoon felis*, unclassified piroplasms (western piroplasms), and *Plasmodium falciparum*. The percentages of 500 bootstrap replications in which groupings appeared are noted above the branches. Piroplasms reported from felids are underlined.

M20025) resulted in an alignment 888 bp in length, of which 497 were invariant, 85 variable characters were parsimony uninformative, and 306 were parsimony informative. Phylogenetic analysis of the partial β-tubulin gene indicated that the panther *Babesia* sp. was related to, but distinct from, *B. odocoilei* and *B. divergens* (Fig. 2).

In earlier studies, the identification of small piroplasms in panthers was based on morphology and induction of fatal cytauxzoonosis in a domestic cat inoculated with panther blood (Butt et al., 1991; Rotstein et al., 1999). Although *C. felis* is morphologically indistinguishable from other small piroplasms, no *Babesia* spp. or other piroplasms have been reported from felids in North America. In this study, molecular characterization of small piroplasms from Florida panthers and translocated Texas cougars indicated that only a small percentage was infected with *C. felis*, and the remaining piroplasm-positive felids were infected with a *Babesia* species.

Based on 18S rRNA gene analysis the *Babesia* sp. detected in Florida panthers was most closely related to a *Babesia* sp. previously detected in *I. ovatus* ticks from Japan (Inokuma et al., 2003), *B. odocoilei* and *B. divergens*. Analysis of β-tubulin gene sequences supported the close relationship with *B. odocoilei* and *B. divergens*. Unfortunately, β-tubulin gene sequences were not available from the Japanese *I. ovatus Babesia* sp. for comparison. This Japanese *Babesia* sp. was detected in three individual ticks collected from domestic dogs; however, the vertebrate host(s) of the Japanese *Babesia* is unknown. The Japanese *Babesia* sp. was initially thought to infect a ruminant

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Table 2. Percent identity between the Florida panther *Babesia* species and selected piroplasms as determined for a portion of the 18S rRNA gene (1,654 bases), which overlapped for all species.

| Organism | Percent identity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Babesia* sp. from panther[a] (DQ329138) | *Babesia* sp. *Fukui766* | *Babesia odocoilei* | *Babesia divergens* | *Babesia canis canis*[a] | *Babesia canis presentii*[a] | *Babesia felis*[a] | *Babesia* sp. from caracal[a] | *Babesia leo*[a] |
| *Babesia* sp. *Fukui766* (AY19012) | 98.6 | | | | | | | | |
| *Babesia odocoilei* (U16369) | 97.4 | 98.1 | | | | | | | |
| *Babesia divergens* (U07885) | 97.0 | 97.5 | 98.0 | | | | | | |
| *Babesia canis canis*[a] (AF205636) | 94.1 | 94.5 | 94.4 | 93.9 | | | | | |
| *Babesia canis presentii*[a] (AY272047) | 94.5 | 94.9 | 94.7 | 93.9 | 95.8 | | | | |
| *Babesia felis*[a] (AF244912) | 88.9 | 89.1 | 89.0 | 88.3 | 87.5 | 87.9 | | | |
| *Babesia* sp. from caracal[a] (AF244913) | 89.1 | 89.4 | 89.0 | 88.5 | 87.5 | 87.8 | 99.3 | | |
| *Babesia leo*[a] (AF244911) | 89.0 | 89.1 | 88.6 | 88.3 | 87.1 | 87.4 | 98.0 | 98.6 | |
| *Cytauxzoon felis*[a] (L19080) | 90.1 | 90.2 | 89.5 | 89.1 | 88.8 | 88.7 | 90.5 | 90.2 | 90.7 |

[a] These species have been reported from felids.

because it is closely related to *B. odocoilei* and *B. divergens*. In general, morphology and phylogenetic placement of piroplasms provides limited information regarding potential hosts; the panther *Babesia* sp. and *B. odocoilei* are small piroplasms, whereas *B. divergens* is a large piroplasm and these three species infect a great diversity of hosts including felids (panther *Babesia* sp.), bovids and several species of cervids (*B. odocoilei*), and cervids, bovids, gerbils, and humans (*B. divergens*) (Lewis and Williams, 1979; Holman et al., 2000; Gray et al., 2002; Duh et al., 2005; Schoelkopf et al., in press).

The vector of the Florida panther *Babesia* sp. is unknown, but *Babesia* species are vectored by ixodid ticks. Five species of adult ticks, *Dermacentor variabilis, Ixodes scapularis, Ixodes affinis, Amblyomma maculatum*, and *Amblyomma americanum*, have been reported to parasitize Florida panthers (Forrester, 1992; Wehinger et al., 1995; Garner et al., 1996). *Dermacentor variabilis* and *I. scapularis* were the two most common ticks present on the panthers. Both of these tick species have a state-wide distribution (Wehinger et al., 1995; Durden et al., 2000; Allan et al., 2001), and nearly 100% of the panthers examined in these studies were infested with at least one of these two tick species. *Amblyomma maculatum* and *A. americanum* were rarely detected on panthers, and the latter tick species was only found on panthers kept in captivity in northern Florida (Wehinger et al., 1995; Garner et al., 1996).

In summary, a *Babesia* species was found in a high percentage of Florida panthers, none of which had evidence of disease compatible with babesiosis (Rotstein et al., 1999); thus it appears to have low pathogenicity for the panthers. The *Babesia* species was closely related to a *Babesia* sp. detected in Japanese *I. ovatus* ticks (vertebrate host unknown). In addition, seven Texas cougars and Florida panthers were positive for *C. felis*, none of which were exhibiting clinical

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100 *Babesia odocoilei* Holman strain Texas White-tailed deer (AY144706)
100 *Babesia odocoilei* Ips01Deer strain Massachusetts White-tailed deer (AY144705)
97 *Babesia* sp. R050401 strain Massachusetts Rabbit (AY144704)
100 *Babesia divergens* Purnell strain Ireland Cow (AY144703)
**Babesia sp. Florida Panther (this study)**
50 Babesia *microti*-like sp. Raccoon (AY144708)
100 *Babesia microti*-like sp. Skunk (AF546902)
*Babesia microti* Korea8 strain (AB083380)
99 *Theileria annulata* Ankara strain (CR940348)
*Toxoplasma gondii* (M20025)
0.05

FIGURE 2. Phylogenetic tree inferred by analysis of β-tubulin gene sequences of the Florida panther *Babesia* sp., related piroplasms, and *Toxoplasma gondii*. The percentages of 500 bootstrap replications in which groupings appeared are noted above the branches.

signs. However, infection with either of these parasites in combination with stress or other factors (e.g., immunosuppression) could possibly lead to disease. An uncharacterized *Babesia* sp. has caused fatal babesiosis in two captive cougars in Egypt that were translocated from California, USA (Carpano, 1934). *Cytauxzoon felis* has caused fatal cytauxzoonosis in the natural reservoir, the bobcat (Nietfeld and Pollock, 2002), and in at least one species of exotic felid (white tiger) in northern Florida, USA (Garner et al., 1996). The importance of the panther *Babesia* species to the health of domestic cats or exotic felids is currently unknown.

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