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Variation in the ITS-1 and ITS-2 rRNA genomic regions of *Cytauxzoon felis* from bobcats and pumas in the eastern United States and comparison with sequences from domestic cats

Barbara C. Shock[a,b,*], Adam J. Birkenheuer[c], Laura L. Patton[d], Colleen Olfenbuttel[e], Jeff Beringer[f], Daniel M. Grove[g], Matt Peek[h], Joseph W. Butfiloski[i], Daymond W. Hughes[j], J. Mitchell Lockhart[k], Mark W. Cunningham[l], Holly M. Brown[m], David S. Peterson[n], Michael J. Yabsley[a,b]

[a] *Southeastern Cooperative Wildlife Disease Study, 589 D.W. Brooks Drive, Wildlife Health Building, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA*
[b] *Warnell School of Forestry and Natural Resources, 180 E Green Street, University of Georgia, Athens, GA 30602, USA*
[c] *NC State College of Veterinary Medicine, 4700 Hillsborough Street, Raleigh, NC 27606, USA*
[d] *Kentucky Department of Fish and Wildlife Resources, 1 Sportsman's Lane, Frankfort, KY 40601, USA*
[e] *North Carolina Wildlife Resources Commission, NCSU Centennial Campus, 1751 Varsity Drive, Raleigh, NC 27606, USA*
[f] *Missouri Department of Conservation, 110 South College Avenue, Columbia, MO 65201, USA*
[g] *North Dakota Game and Fish Department, 100 N. Bismarck Expressway, Bismarck, ND 58501, USA*
[h] *Kansas Department of Wildlife and Parks, 512 SE 25th Avenue, Pratt, KS 67124, USA*
[i] *South Carolina Department of Natural Resources, 1000 Assembly Street, Columbia, SC 29201, USA*
[j] *United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, 2024 Newton Road, Albany, GA 31701, USA*
[k] *Biology Department, Valdosta State University, 1500 N Patterson, Room 2035, Valdosta, GA 31698, USA*
[l] *Florida Fish and Wildlife Conservation Commission, 1105 Southwest Williston Road, Gainesville, FL 32601, USA*
[m] *Department of Pathology, 501 D.W. Brooks Drive, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA*
[n] *Department of Infectious Diseases, 501 D.W. Brooks Drive, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA*

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ABSTRACT

Cytauxzoon felis, a tick-borne protozoan parasite, is the causative agent of cytauxzoonosis in domestic cats in the United States. The natural reservoir for this parasite is the bobcat (*Lynx rufus*), which typically does not develop clinical signs. Although not likely important reservoirs, *C. felis* has also been detected in pumas (*Puma concolor*) in Florida and Louisiana. Recent studies suggest that specific genotypes of *C. felis* that circulate in domestic cats may be associated with variable clinical outcomes and specific spatial locations. In the current study, we investigated the intraspecific variation of the *C. felis* internal transcribed spacer (ITS)-1 and ITS-2 rRNA regions from 145 wild felids (139 bobcats and six pumas) from 11 states (Florida, Georgia, Kansas, Kentucky, Louisiana, Missouri, North Carolina, North Dakota, South Carolina, Oklahoma, and Pennsylvania). Unambiguous ITS-1 and ITS-2 data were obtained for 144 and 112 samples, respectively, and both ITS-1 and ITS-2 sequences were obtained for 111 (77%) samples. For the ITS-1 region, sequences from 65 samples collected from wild felids were identical to those previously reported in domestic cats, while the other 79 sequences were unique. *C. felis* from 45 bobcats and one puma had ITS-1 sequences identical to the most common sequence reported from domestic cats. Within the ITS-2 region, sequences from 49 bobcats were identical to those previously reported in domestic cats and 63 sequences were unique (with some occurring in more than one

* Corresponding author at: 589 DW Brooks Drive, Wildlife Health Building, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA. Tel.: +1 706 542 1741; fax: +1 706 542 5865.
E-mail addresses: bshock@uga.edu, barbarashock@gmail.com (B.C. Shock).

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bobcat). The most common ITS-2 sequence from domestic cats was also common in wild felids (31 bobcats and a puma). Samples from three pumas from Florida and two bobcats from Missouri had a 40- or 41-bp insert in the ITS-2 similar to one described previously in a domestic cat from Arkansas. Additionally, a previously undescribed 198- or 199-bp insert was detected in the ITS-2 sequence from four bobcats. Collectively, based on combined ITS-1 and ITS-2 sequences, five different genotypes were detected in the wild felids. Genotype ITSa was the most common genotype (11 bobcats and one puma) and fewer numbers of ITSb, ITSe, ITSg, and ITSi were detected in bobcats. These data indicate that, based on ITS-1 and ITS-2 sequences, numerous *C. felis* strains may circulate in wild felids.

1. Introduction

Cytauxzoon felis, a tick-borne protozoan parasite, is the causative agent of cytauxzoonosis in domestic cats in the United States. First identified in domestic cats (*Felis silvestris catus*) from Missouri, Texas, and Arkansas in the 1970s (Bendele et al., 1976; Wagner, 1976; Wightman et al., 1977), *C. felis* has subsequently been identified in domestic cats from numerous southeastern, midwestern, and mid-Atlantic states (Wagner, 1976; Ferris, 1979; Kier et al., 1982b; Birkenheuer et al., 2006). In addition to domestic cats, infections of *C. felis* have been determined in bobcats (*Lynx rufus*) and pumas (*Puma concolor*).

In domestic cats, *C. felis* can cause erythrocyte hemolysis and occlusion of the lumen of blood vessels by large schizont-laden mononuclear phagocytes in the lungs, liver, lymph nodes, and spleen (Simpson et al., 1985; Kier et al., 1987; Kocan and Kocan, 1991; Kocan et al., 1992). Historically, infection with this parasite was believed to be nearly uniformly fatal (Ferris, 1979); however, recent studies have discovered an increasing number of domestic cats that have subclinical chronic infections (Haber et al., 2007; Birkenheuer et al., 2006). Both *Amblyomma americanum* (lone star tick) and *Dermacentor variabilis* (American dog tick) are confirmed vectors, and both tick species are common in the southeastern and midwestern United States where the majority of *C. felis* infections have been identified (Blouin et al., 1984; Kocan et al., 1992; Reichard et al., 2008, 2010; Shock et al., 2011).

The bobcat (*L. rufus*) is considered to be the primary wildlife reservoir. In bobcats, prevalences of >30% have been documented in populations from several states: Arkansas, Florida, Kansas, Kentucky, Missouri, Oklahoma, North Carolina, and South Carolina (Wagner, 1976; Glenn et al., 1983; Birkenheuer et al., 2008; Brown et al., 2010; Shock et al., 2011), while lower prevalences were observed in bobcats sampled from Pennsylvania and North Dakota (Birkenheuer et al., 2008; Shock et al., 2011). Experimental and field-based studies indicate that the majority of infected bobcats have subclinical infections; however, rare cases of mortality have been observed in experimentally and naturally infected bobcats (Kier et al., 1982a, 1982b; Glenn et al., 1983; Blouin et al., 1984, 1987; Nietfeld and Pollock, 2002). The life stage of the parasite used in the inoculum during experimental trials seems to be important for disease outcome. Bobcats inoculated with schizogenous stages of the parasite died of acute cytauxzoonosis, while bobcats experimentally infected by a natural route (i.e., tick transmission) developed a limited schizogenous phase, which led to long-term subclinical parasitemia (Kier et al., 1982b; Blouin et al., 1987).

Natural infections have been reported from a puma from Louisiana and Florida pumas (*P. concolor coryi*) (Butt et al., 1991; Yabsley et al., 2006; Shock et al., 2011). In contrast to the bobcat, *C. felis* appears to cause a mild, likely transient, hemolytic anemia in Florida pumas (Harvey et al., 2007). Worldwide, other *Cytauxzoon* spp. that are distinct from *C. felis* have been identified in numerous wild felid species (Butt et al., 1991; Luaces et al., 2005; Peixoto et al., 2007; Ketz-Riley et al., 2003).

Several treatment options have been investigated for domestic cats and a combination treatment with atovaquone and azithromycin increased survival compared with treatment with imidocarb dipropionate alone (Cohn et al., 2011). However, increasing reports of subclinical infections that appear to be unrelated to treatment could be due to (1) emergence of different strains of *C. felis* that may differ in their virulence for domestic cats, (2) some selection of nonpathogenic strains by domestic cat survival which may be maintained in subsequent generations or (3) use of more sensitive diagnostic tests (e.g. PCR) which has led to increased detection (Birkenheuer et al., 2006; Haber et al., 2007; Brown et al., 2008).

Currently, there is no data on virulence genes for *C. felis*, but four recent studies have examined genetic variation within the internal transcribed spacer (ITS) regions of the ribosomal RNA genes (Brown et al., 2009a, 2009b, 2010; Cohn et al., 2011). In the initial characterization paper, significant spatial correlations and associations with clinical outcome were associated with specific genotypes (Brown et al., 2009a); however, subsequent studies failed to find an association (Brown et al., 2009b, 2010; Cohn et al., 2011). Only a single study has genetically characterized *C. felis* strains from bobcats, but this study was limited to 25 *C. felis* samples from bobcats from Arkansas, Florida, and Georgia (Brown et al., 2010). Of the 11 *C. felis* genotypes detected in bobcats, only three had been previously identified in domestic cats. The current study aims to further understand the sylvatic cycle of *C. felis* and to more robustly characterize the strains of *C. felis* circulating in wild felids. Because bobcats are the natural reservoir, we hypothesize that additional genetic variability will be identified in *C. felis* strains from wild felids and that spatial correlations with genotype will be more easily identified because of the high prevalence of *C. felis* infections in these reservoirs and the lack of movement of wild felids to new geographic regions compared to domestic cat human-associated movement.

2. Materials and methods

2.1. Samples

DNA samples from 161 *C. felis*-infected bobcats ($n = 153$) and pumas ($n = 8$) from previous surveys on wild felids were included in this study (Yabsley et al., 2006; Birkenheuer et al., 2008; Shock et al., 2011). These samples were confirmed positive for *C. felis* by either amplification of the 18S rRNA gene or the ITS-1 region followed by sequence analysis (Yabsley et al., 2006; Shock et al., 2011). Based on ITS-1 sequences, *C. felis* can be distinguished from other *Cytauxzoon* spp. and from other closely related piroplasms (*Babesia* and *Theileria* spp.) by analysis of only ITS-1 sequences (Shock et al., unpublished). All DNA samples were maintained at −20 °C or −80 °C until testing in the current study.

2.2. Genetic characterization

Two different regions were targeted to identify genetic variability, the ITS-1 and ITS-2 regions. The ITS-1 region was amplified using a nested PCR that amplifies this genetic region from the piroplasms *Cytauxzoon*, *Babesia*, and *Theileria* spp. (Bostrom et al., 2008). Briefly, for primary amplification, 5 μl of DNA was added to 20 μl of a master mix containing 10 mM Tris–Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Promega, Madison, WI), 2.5 units GoTaq® Flexi DNA Polymerase (Promega), and 0.8 μM of primers ITS-15C (5′-CGATCGAGTGATCCGGTGAATTA) and ITS-13B (5′-GCTGCGTCCTTCATCGTTGTG). Cycling parameters were 94 °C for 1 min followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 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 inclusion of primers ITS-15D (5′-AAGGAAGGAGAAGTCGTAACAAGG) and ITS-13C (5′-TTGTGTGAGCCAAGACATCCA). The cycling parameters were the same as the primary reaction except the annealing temperature was 49 °C. A single PCR was used to amplify the ITS-2 region that can amplify the ITS-2 region from *Cytauxzoon* spp. and *Babesia* spp., but not other closely related Apicomplexans. The same master mix protocol was used except for the inclusion of primers FOR7 (5′-AGCCAATTGCGATAAGCATT) and REV7 (5′-TCACTCGCCGTTACTAGGAGA) and the cycling parameters were 96 for 3 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min 30 s, and a final extension at 72 °C for 7 min. Additionally, a nested PCR that was used to amplify the continuous rRNA region (18S–28S) for 51 samples, which were also run with the above primers. This PCR can amplify sequences from *Babesia* spp. and *Cytauxzoon* spp. For primary amplification, 5 μl of DNA was added to 20 μl of a master mix containing 10 mM Tris–Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM each dNTP (Promega, Madison, Wisconsin), 2.5 units Taq DNA Polymerase (Promega), and 0.8 μM of primers 1055F (5′-GGTGGTGCATGGCCG) and ITSR (5′-GGTCCGTGTTTCAAGACGG). Cycling parameters were 96 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 90 s, and a final extension at 72 °C for 7 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 inclusion of primers LSUR300 (5′-TWGCGCTTCAATCCC) and ITSF (5′-GAGAGAGAAGTCGTAACAAGGTTTCCG). Cycling parameters were the same as the primary (Yabsley et al., 2009).

To prevent and detect contamination, primary and secondary amplification, and product analysis were done in separate dedicated areas. A negative water control was included in each set of DNA extraction, and one water control was included in each set of primary and secondary PCR reactions. All amplicons of the appropriate size (~550 bp for ITS-1 and ~300 bp for ITS-2) were purified with a Qiagen gel extraction kit (Germantown, MD) and bi-directionally sequenced at the University of Georgia Integrated Biotechnology Laboratory (Athens, GA).

Chromatogram data were analyzed using Sequencher (Ann Arbor, MI). Sequences obtained from this study and available in GenBank were aligned using the multisequence alignment ClustalX program within MEGA (Molecular Evolutionary Genetics Analysis) version 3.1 program (Kumar et al., 2004).

3. Results

Amplification and sequencing of the two ITS regions of *C. felis* from samples previously determined to be infected with *C. felis* yielded ITS-1 and/or ITS-2 data for 145 of 161 (90%) samples. ITS-1 sequence data was obtained for 144 of 161 (89%) samples from 11 states and ITS-2 sequence data for 112 of 161 (69%) samples from 10 states (Table 1). Combined ITS-1 and ITS-2 sequence data was available for 111 of the 145 sequenced samples (77%). Sequences of the ITS-1, 5.8S rRNA, and ITS-2 obtained from the 51 samples using the 1055F/ITSR and LSUR300/ITSF primers were identical to individual ITS-1 and ITS-2 sequences obtained by the two genome region specific reactions. Assignment of samples to a particular genotype was only conducted on samples that had both ITS-1 and ITS-2 data (as described by Brown et al., 2009a, 2009b, 2010). Genotype designations and corresponding GenBank accession numbers are shown in Tables 2 and 3.

Within the 458-bp ITS-1 region, there were 100 single nucleotide polymorphisms (SNPs) or insertions/deletions. The most common ITS-1 sequence detected in this study was identical to EU450802 and was found in 45 bobcats from Florida, Georgia, Kansas, Kentucky, Missouri, North Carolina, and Oklahoma and one puma from Louisiana (Table 1). The second most common ITS-1 sequence (GU581167) was detected in 17 bobcats from Florida ($n = 11$) and Georgia ($n = 6$). Novel ITS-1 sequences were detected in 79 bobcat *C. felis* samples (Table 1). Three of the novel sequences were found in multiple bobcats; two sequences from Oklahoma and one from Kentucky were identical to each other (HQ383872), one sequence each from Kansas and North Carolina were identical (HQ383856), and one sequence each from Florida and Georgia were identical (HQ383871). The remaining 72 sequences were unique (HQ383813–HQ383855; HQ383857–HQ383870; JF308486–JF308499) (Table 1).

Table 1
Number and distribution of *Cytauxzoon felis* ITS-1 and ITS-2 sequences types among wild felids.

GenBank accession no.	*n* (% of total)	Number of each genetic type by state of origin										
		FL	GA	KS	KY	LA	MO	NC	ND	SC	OK	PA
ITS1												
EU450802[a]	46 (32)	2	2	4	20	1	8	7	0	0	2	0
GU581166[c]	1 (0.7)	0	0	0	1	0	0	0	0	0	0	0
GU581167[c]	17 (12)	11	6	0	0	0	0	0	0	0	0	0
GU581169[c]	1 (0.7)	1	0	0	0	0	0	0	0	0	0	0
HQ383871[d]	2 (1)	1	1	0	0	0	0	0	0	0	0	0
HQ383856[d]	2 (1)	0	0	1	0	0	0	1	0	0	0	0
HQ383872[d]	3 (2)	0	0	0	1	0	0	0	0	0	2	0
Unique[d,e]	72 (50)	5	2	6	15	0	21	7	3	2	9	2
Total number of different genetic types	79	9	5	8	18	1	22	9	3	2	11	2
ITS2												
EU450804[a]	32 (29)	2	1	0	9	1	12	1	1	0	5	0
EU450805[a]	1 (1)	0	1	0	0	0	0	0	0	0	0	0
FJ536419[a]	3 (3)	0	1	0	1	0	1	0	0	0	0	0
GU581170[c]	9 (8)	5	4	0	0	0	0	0	0	0	0	0
GU581171[c]	3 (3)	3	0	0	0	0	0	0	0	0	0	0
FJ536418[b]	1 (1)	0	0	0	0	0	0	0	0	1	0	0
HQ383917[d]	5 (5)	0	0	0	5	0	0	0	0	0	0	0
HQ383918[d]	3 (3)	0	0	0	1	0	1	1	0	0	0	0
HQ383919[d]	2 (2)	0	0	0	2	0	0	0	0	0	0	0
Unique[d,f]	53 (47)	10	1	2	15	0	11	9	0	1	2	2
Total number of different genetic types	62	13	5	2	20	1	14	11	1	2	3	2

[a] Designates sequences that were reported in Brown et al. (2009a).
[b] Designates sequences that were reported in Brown et al. (2009b).
[c] Designates sequences that were reported in Brown et al. (2010).
[d] Designates sequences that are novel and first reported in the current study.
[e] HQ383813–HQ383855; HQ383857–HQ383870; JF308486–JF308499.
[f] HQ383873–HQ383876; HQ383879; HQ383882–HQ383896; HQ383898–HQ383907; HQ383909–HQ383916; JF308500–JF308509.

Table 2
Geographic origin of different genotypes of *Cytauxzoon felis*.

Genotype	ITS1 accession no.	ITS2 accession no.	State of origin							
			FL	GA	KY	LA	MO	NC	OK	Total
ITSa	EU450802	EU450804	1	0	3	1	4	1	2	12
ITSb	EU450802	EU450805	0	1	0	0	0	0	0	1
ITSe	GU581167	GU581170	5	3	0	0	0	0	0	8
ITSg	GU581167	GU581171	2	0	0	0	0	0	0	2
ITSi	EU450802	FJ536419	0	1	1	0	0	0	0	2

Within the 265-bp ITS-2 region of *C. felis* (excluding the two insertions mentioned below), there was a total of 184 SNPs, 10 single nucleotide insertions, and 12 single nucleotide deletions. The most common sequence was identical to EU450804 and was found in *C. felis* from 31 bobcats from North Carolina, North Dakota, Florida, Oklahoma, Kentucky, and Missouri and in *C. felis* from the one puma from Louisiana. Five other previously reported ITS-2 sequences (EU450805, FJ536418, FJ536419, GU581170, and GU581171) were detected in *C. felis* from 17 bobcats (Table 1). Novel ITS-2 sequences were detected in 62 bobcat *C. felis* samples (Table 1). Three of the novel ITS-2 sequences were detected in *C. felis* from five bobcats from Kentucky (HQ383917), three bobcats from North Carolina, Missouri and Kentucky (HQ383918), and two bobcats from Kentucky (HQ383919). The remaining 52 sequences were unique (HQ383873–HQ383876; HQ383879; HQ383882–HQ383896; HQ383898–HQ383907; HQ383909–HQ383916; JF308500–JF308509) (Table 1).

Three ITS-2 sequences from *C. felis* from Florida pumas (HQ383878, HQ383880, HQ383881) had a 40 bp insert nearly identical (38 or 39 of 40 bases) to an insert in a sequence from *C. felis* from a single domestic cat (EU450806) from Arkansas; however, numerous SNPs within the non-insert region of the ITS-2 distinguished these sequences from EU450806. An additional sample from a bobcat from Missouri (HQ383897) had a 41 bp insert at the sample location as EU450806 and the samples from the Florida pumas. Two samples (HQ383908, HQ383909) from bobcats from North Carolina had a 199 bp insert after position 152 (EU450804) that were 94.4% identical to each other. Two additional bobcats from Pennsylvania and Kentucky (HQ383877; JF330260) had a similar 198 bp insert (98.9% identical to each other and 94.9–95.9% similar to the North Carolina samples). Sequence analysis of the 18S rRNA gene for these samples with unique inserts confirmed their identity as *C. felis*.

Table 3
Designation of genotypes and corresponding GenBank accession numbers for *Cytauxzoon felis* detected in domestic cats and wild felids.

GenBank accession no.		Brown et al. (2009a)		Brown et al. (2009b)		Brown et al. (2010)			Current study		
ITS1	ITS2	Designation	Domestic	Designation	Domestic	Designation	Domestic	Bobcat	Designation	Bobcat	Puma
EU450802	EU450804	ITSA	48	ITSc	3	ITSa	16	5	ITSa	11	1
EU450802	EU450805	ITSB	21	ITSb	8	ITSb	8	0	ITSb	1	0
EU450803	EU450804	ITSC	5			ITSc	1	0	ITSc	0	0
GU581166	EU450805					ITSd	0	1	ITSd	0	0
GU581167	GU581170					ITSe	0	1	ITSe	8	0
EU450802	FJ536421	ITSc	2	ITSf	1	ITSf	0	1	ITSf	0	0
GU581167	GU581171					ITSg	0	8	ITSg	2	0
EU450802	GU581172					ITSh	0	1	ITSh	0	0
EU450802	FJ536419	ITSd	4	ITSd	3	ITSi	0	3	ITSi	2	0
FJ536425	EU450804					ITSj	0	2	ITSj	0	0
FJ536425	GU581171					ITSk	0	1	ITSk	0	0
GU581168	GU581171					ITSl	0	1	ITSl	0	0
GU581169	GU581171					ITSm	0	1	ITSm	0	0
EU450802	EU450806	ITSg	1						ITSn	0	0
EU450802	FJ536418	ITSh	1	ITSa	27				ITSo	0	0
EU450802	FJ536420			ITSe	1				ITSp	0	0
FJ536423	FJ536418			ITSg	1				ITSq	0	0
FJ536424	FJ536418			ITSh	1				ITSr	0	0
FJ536425	FJ536418			ITSi	1				ITSs	0	0
FJ536426	FJ536418			ITSj	1				ITSt	0	0
EU450802	FJ536422			ITSk	1				ITSu	0	0
FJ536425	FJ536422	ITSe	2						ITSv	0	0

All capital letters in the designation indicates what was previously labeled a genotype.

Genotypes have previously been determined using a combination of ITS-1 and ITS-2 sequences (Brown et al., 2009a, 2009b, 2010). When the ITS-1 and ITS-2 sequences were combined, 25 samples had genotypes that had previously been described. Twelve wild felids from numerous states were infected with the *C. felis* ITSa genotype (Table 2) which is the most commonly reported genotype among domestic cats (Brown et al., 2009a, 2010). Only four other genotypes (ITSb, ITSe, ITSg, and ITSi) were detected in 13 other bobcats (Table 2). Because of inconsistent genotype designations used in previous studies, we present a consensus of data regarding these previously detected genotypes in domestic cats and wild felids combined with new data presented in the current study in Table 3. For simplicity, all previously reported genotypes and sequences types are consolidated into genotype designations that include ITSa–ITSv (Table 3). Temporal analysis of our data from bobcats collected from 1999 to 2010 revealed no association between year and genotype or state (data not shown).

4. Discussion

In the current study, we characterized the genetic variation in the ITS-1 and ITS-2 regions of *C. felis* from wild felids, primarily bobcats, from numerous states in the eastern and central United States. Compared with the four previous studies which were conducted primarily on *C. felis* sequences obtained from domestic cats (Brown et al., 2009a, 2009b, 2010; Cohn et al., 2011), we observed more polymorphisms in both of these genetic targets including SNPs and insertions/deletions. This greater diversity could be due to a larger sample size, or more likely, because wild felids are the reservoir for this parasite. This increased genetic diversity is not surprising in the natural reservoir as infections have been documented as early as 1930 (Wenyon and Hamerton, 1930) and some studies suggest that non-coding variation may be due to host–parasite co-evolution (Rosenthal, 2001; Maizels and Kurniaqan-Atmadja, 2002; Matrajt, 2010). In addition to being the primary reservoir, bobcats are useful for this type of project because they are potentially exposed to higher numbers of infected ticks compared with domestic cats and prevalences in bobcat populations in some regions may exceed 50% which provides greater numbers of parasites for characterization at particular geographic locations (Shock et al., 2011).

Sequence analysis was complicated due to bobcats being co-infected with multiple *C. felis* strains or the presence of multiple rRNA copies in the genome; however, the percentages of unambiguous sequences obtained for this study were similar to those obtained in previous studies in domestic cats and bobcats (Brown et al., 2009b, 2010). Some polymorphisms may have been incorporated because we did not use a high-fidelity *Taq* polymerase, although the Taq polymerase used in this study has a lower reported error rate (0.00001, Promega) than the polymerase used in previous studies (Brown et al., 2009a, 2010). Future work conducted with the ITS-1 and ITS-2 regions should include cloning of projects prior to sequencing; however, whole genome sequencing may be the best technique understand *C. felis* genetic variability and will provide additional gene targets for virulence research.

The first study to genetically characterize a large number of *C. felis* samples was conducted on samples from domestic cats from Arkansas and Georgia (Brown et al., 2009a). The study found a possible association between geographic location and genotype because a significant

proportion (84%) of the *C. felis* samples from Arkansas were identified as genotype ITSa, and the majority of samples (68%) from Georgia were classified as genotype ITSb (Brown et al., 2009a). However, a second study that included samples only from Georgia found that ITSo (our genotype designation, initially reported as ITSa, Table 3) was the predominate genotype followed by genotype ITSb (Brown et al., 2009b, Table 3). Genotype ITSo has not been found in subsequent studies (Brown et al., 2009b, 2010; Cohn et al., 2011, current study). Interestingly, genotype ITSo is very similar to genotype ITSa because both have identical ITS-1 sequences and the ITS-2 sequences only differ by one nucleotide. Similarly, genotypes ITSa and ITSb have identical ITS-1 sequences and can only be differentiated based on ITS-2; ITSb has a thymine at nucleotide position 180 (EU450805; Table 3).

Similar to two previous studies (Brown et al., 2009a, 2010), genotype ITSa was the most common genotype that we detected in wild felids and it was found across a wide geographic range. In contrast, we only detected the ITSb genotype in a single bobcat from Georgia whereas this genotype was very common in domestic cats from Georgia (Brown et al., 2009a, 2009b, 2010). This may be due to sample location differences or ITSb may preferentially infect domestic cats. Interestingly, the ITS-1 sequence (EU450802) that was most common in previous studies in both domestic cats and bobcats was the most common ITS-1 sequence detected in the wild felids included in our study. Similarly, the most common ITS-2 sequence (EU450804) from our study was also the most common sequence detected in domestic and bobcats (Brown et al., 2009a, 2009b, 2010).

One single genotype detected in wild felids appeared to be restricted to a single location. Genotype ITSe was only found in bobcats from northern Florida and southern Georgia. This genotype was previous reported by Brown et al. (2010), also from bobcats from the same area. This area represents one of the few places where we were able to obtain large numbers of bobcat samples from a localized area which likely increased our chances of detecting gentotypes that are correlated with localized areas. The remaining samples from the study were opportunistically collected, both spatially and temporally. This resulted in few individuals from individual locations or time periods which may explain why novel sequence types of both ITS-1 and ITS-2 regions were detected, but no novel genotypes were detected. If we were able to obtain more samples from wild felids in the areas during the same time periods we may have observed more spatial and temporal correlations. Additional samples from both wild and domestic felids from geographically discrete areas are needed to better understand the genetic variability of *C. felis* and if these genetic types are associated with specific geographic regions or hosts. For example, a more extensive genetic characterization of *C. felis* infections from the increasing number of infected domestic cats would be beneficial.

Neither of the sequenced regions examined in the current study, or previous studies (Brown et al., 2009a, 2009b, 2010), are related to virulence genes or any potential protein involved in pathogenicity. They are simply being used as potential markers for observed biological or spatial differences. The data from this study of *C. felis* from wild felids clearly show that the sequence variability in these regions is very high, but may be of limited value for studies of virulence or even geographic clustering. To better understand the role of bobcats, or chronically infected domestic cats, in maintaining virulent strains of *C. felis*, future studies should characterize potential homologues of genes (e.g., variant erythrocyte surface antigen-1 (VESP1), leucine aminopeptidase, or heat shock protein-70 proteins) associated with pathogenicity or antigenic variation in related pathogens such as *Babesia* or *Theileria* (Yamasaki et al., 2007; Jia et al., 2009; Xiao et al., 2010).

Conflict of interest statement

The authors have no conflict of interest.

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