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Molecular Detection of *Rickettsia felis* in Different Flea Species from Caldas, Colombia

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Abstract. Rickettsioses caused by *Rickettsia felis* are an emergent global threat. Historically, the northern region of the province of Caldas in Colombia has reported murine typhus cases, and recently, serological studies confirmed high seroprevalence for both *R. felis* and *R. typhi*. In the present study, fleas from seven municipalities were collected from dogs, cats, and mice. DNA was extracted and amplified by polymerase chain reaction (PCR) to identify *gltA*, *ompB*, and *17kD* genes. Positive samples were sequenced to identify the species of *Rickettsia*. Of 1,341 fleas, *Ctenocephalides felis* was the most prevalent (76.7%). Positive PCR results in the three genes were evidenced in *C. felis* (minimum infection rates; 5.3%), *C. canis* (9.2%), and *Pulex irritans* (10.0%). Basic Local Alignment Search Tool (BLAST) analyses of sequences showed high identity values (> 98%) with *R. felis*, and all were highly related by phylogenetic analyses. This work shows the first detection of *R. felis* in fleas collected from animals in Colombia.

INTRODUCTION

Bacteria from the genus *Rickettsia* are obligate intracellular microorganisms transmitted by arthropods to vertebrate hosts, including man and domestic animals.[1] *R. felis* infection in humans produces a disease known as flea-borne spotted fever (or cat flea typhus), which is an emergent and global threat.[2] Despite reports of infection in almost 12 different species of fleas, 8 species of ticks, mites, and lice, the cat flea (*Ctenocephalides felis felis*) is currently the only arthropod associated with the biological transmission of this agent.[3,4]

R. felis has been identified in fleas and other arthropods from different countries in the Americas, including Argentina,[5] Brazil,[6–11] Canada,[12] Chile,[13] Costa Rica,[14] Mexico,[15] Panama,[16] Peru,[17] the United States,[18–22] and Uruguay.[23]

The northern aspect of Caldas Province, Colombia, is an area that historically reports murine typhus cases to the public health authorities. Previous studies in this region confirmed (by indirect fluorescence assay [IFA]) cases of rickettsioses that were seropositive for anti-*R. typhi* immunoglobulin G (IgG) and IgM.[24] Recently, we completed a transversal serological study in seven municipalities of this region and found seroprevalence of 25.2% and 17.8% against *R. typhi* and *R. felis*, respectively. A prospective arm of this study also corroborated different human infections with the aforementioned flea-borne rickettsial species.[25]

The aim of this work was to detect, by molecular methods, the presence of *Rickettsia* species in fleas collected from animals in the urban area of seven municipalities from Caldas Province, Colombia.

MATERIALS AND METHODS

Geographical location. Municipalities included in the study are listed in Tables 1 and 2 and highlighted in the map (Figure 1 and Supplemental Figure 1).

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Fleas. Fleas were collected manually or by hair combing from owned dogs and cats. They were also collected from synanthropic rats and mice in seven municipalities from the north of Caldas between 2010 and 2011. All specimens were conserved in 70% ethanol and further classified by current morphological keys.[26,27]

DNA extraction. We produced pools of one to seven fleas from the same species, host, and site of sampling as pooling criteria. Each pool was dried in 70% ethanol in a bath at 70°C and subsequently cut into small fragments on sterile filter paper. The pieces were macerated in 40 μL (1×) phosphate-buffered saline solution (PBS) and stored at −20°C. We used a commercial kit (DNeasy Blood and Tissue; QIAGEN Inc., Valencia, CA) for DNA extraction with the addition of 400 μL guanidine-thiocyanic acid (DNAzol; Invitrogen™, Life Technologies Corp., Grand Island, NY) for tissue lysis. All extracted samples were evaluated in a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Walthman, MA) for DNA concentration and purity, and they were conserved at −20°C for additional analyses.

Molecular detection. DNA of each pool was amplified by conventional polymerase chain reaction (PCR) with specific primers for *gltA* (CS78–CS323; 401 bp)[28] *Rickettsia* gene, and all positive samples were further confirmed with primers for *ompB* (120M59-120.807; 862 bp)[29] and *17kD* (17kD1–17kD2; 434 bp)[30] genes. For each reaction, a positive control (*R. rickettsii* DNA, infected Vero cells, and Sheila Smith strain) and a negative control (water) were added to the procedure. PCR reactions were performed in a C1000 Thermal Cycler (Bio-Rad Lab., Hercules, CA) with the original conditions reported for each set of primers mentioned above; 10 μL of the PCR products were separated in a 2.0% agarose gel stained with SYBRsafe (Invitrogen™, Life Technologies Corp., Grand Island, NY) and examined in an ultraviolet transilluminator.

Sequencing and analyses. Samples selected for sequencing were PCR-amplified using a proofreading *Taq* polymerase system (Expand High Fidelity PLUS PCR System; Roche, Pleasanton, CA) for each of the three pair of primers described above. Thereafter, all products were purified with

TABLE 1
Fleas and numbers of pools positive for the *Rickettsia* genes *gltA*, *ompB*, and *17kD* collected from animals in seven municipalities from Caldas, Colombia

Municipality	Number of fleas collected				Total number of fleas (number of pools)		Number of positive pools (MIR; %)					
	Dogs (276)	Cats (63)	Rats (6)	Mice (7)			*gltA*		*ompB*		*17kD*	
Aguadas												
C. felis	117	9	–	–	126	(15)	14	(11.1)	14	(11.1)	12	(9.5)
C. canis	38	–	–	–	38	(5)	4	(10.5)	3	(7.9)	4	(10.5)
P. irritans	5	–	–	–	5	(1)	–		–		–	
X. cheopis	1		–	–	1	(1)	–		–		–	
Aranzazu												
C. felis	62	53	–	–	115	(15)	13	(11.3)	13	(11.3)	11	(9.6)
C. canis	36	2	–	–	38	(6)	4	(10.5)	4	(10.5)	3	(7.9)
P. irritans	6	–	–	–	6	(1)	1	(16.7)	1	(16.7)	1	(16.7)
X. cheopis	–	–	–	–	–	(–)	–		–		–	
Filadelfia												
C. felis	154	72	–	–	226	(31)	8	(3.5)	6	(2.7)	6	(2.7)
C. canis	7	4	–	–	11	(3)	3	(27.3)	3	(27.3)	1	(9.1)
P. irritans	2	–	–	–	2	(1)	1	(50)	–		–	
X. cheopis	1	–	10	–	11	(1)	–		–		–	
La Merced												
C. felis	81	27	–	–	108	(15)	5	(4.6)	–		4	(3.7)
C. canis	1	–	–	–	–	(–)	–		–		–	
P. irritans	–	–	–	–	–	(–)	–		–		–	
X. cheopis	–	–	–	–	–	(–)	–		–		–	
Neira												
C. felis	267	16	–	–	283	(35)	30	(10.6)	28	(9.9)	21	(7.4)
C. canis	87	10	1	–	98	(12)	10	(10.2)	10	(10.2)	10	(10.2)
P. irritans	13	1	–	–	14	(3)	–		–		–	
X. cheopis	–	–	–	–	–	(0)	–		–		–	
Pácora												
C. felis	33	14	–	–	47	(6)	6	(12.8)	5	(10.6)	4	(8.5)
C. canis	66	4	–	–	70	(9)	9	(12.9)	8	(11.4)	7	(10.0)
P. irritans	1	–	–	–	1	(1)	–		–		–	
X. cheopis	–	–	–	–	–	(–)	–		–		–	
Salamina												
C. felis	93	30	–	–	123	(15)	12	(9.8)	8	(6.5)	6	(4.9)
C. canis	5	1	–	–	6	(2)	2	(33.3)	1	(16.7)	1	(16.7)
P. irritans	3	1	3	–	7	(3)	1	(14.3)	–		–	
X. cheopis	–	–	–	4	4	(1)	–		–		–	
Total					1,341	(182)	123	(9.2)	104	(7.8)	91	(6.8)

a commercial kit (Wizard SV Gel and PCR Clean-Up System; Promega Corp., Madison, WI) and sequenced (3500 Genetic Analyzer; Applied Biosystems®, Life Technologies Corp., Grand Island, NY). The nucleotide chromatograms were edited with BIOEDIT software (http://www.mbio.ncsu.edu/bioedit/bioedit.html), and sequences were compared with other available *Rickettsia* sequences from GenBank using the BLAST tool.[31]

Phylogenetic analyses. Evolutionary history was inferred by using the maximum likelihood method based on the Tamura–Nei model.[32] Trees for each of the three genes (*gltA*, *ompB*, and *17kD*) were obtained by inference from 1,000 replicates (Figures 1–3). All evolutionary analyses were conducted in MEGA 5.[32]

TABLE 2
Total fleas collected by host and in all locations

	Total fleas collected by host				Total fleas collected (all municipalities)
	Dogs	Cats	Rats	Mice	
C. felis	807	221	–	–	1,028
C. canis	240	21	1	–	262
P. irritans	30	2	3	–	35
X. cheopis	2	–	10	4	16
Total	1,079	244	14	4	1,341

Ethical guidelines. This study was approved by the Pontificia Universidad Javeriana ethical committee and complies with the National Research Council guidelines.

RESULTS

A total of 1,341 fleas were collected in all seven localities. *C felis* was the most prevalent species, with 1,028 (76.7%) specimens, followed by *C. canis* (262; 19.5%), *Pulex irritans* (35; 2.6%), and *Xenopsylla cheopis* (16; 1.2%); 1,079 (80.5%) of these fleas were from dogs, 244 (18.2%) fleas were from cats, 14 (1%) fleas were from rats, and 4 (0.3%) fleas were from mice (Tables 1 and 2).

A total of 182 pools was produced and included in the amplification. Minimum infection rates (MIRs) were calculated as the percentage of a ratio between the total number of flea pools positive for *R. felis* and the total number of fleas tested.[33] We made this assessment with the assumption that only one flea from each positive pool was positive for the *Rickettsia* gene analyzed. A total of 123 (MIR; 9.2%) pools yielded PCR products of the expected size for *gltA*, 104 (7.8%) pools yielded PCR products of the expected size for *ompB*, and 91 (6.8%) pools yielded PCR products of the expected size for *17kD* gene (Tables 1 and 2); 79 (5.9%) pools were positive for the three genes evaluated from all

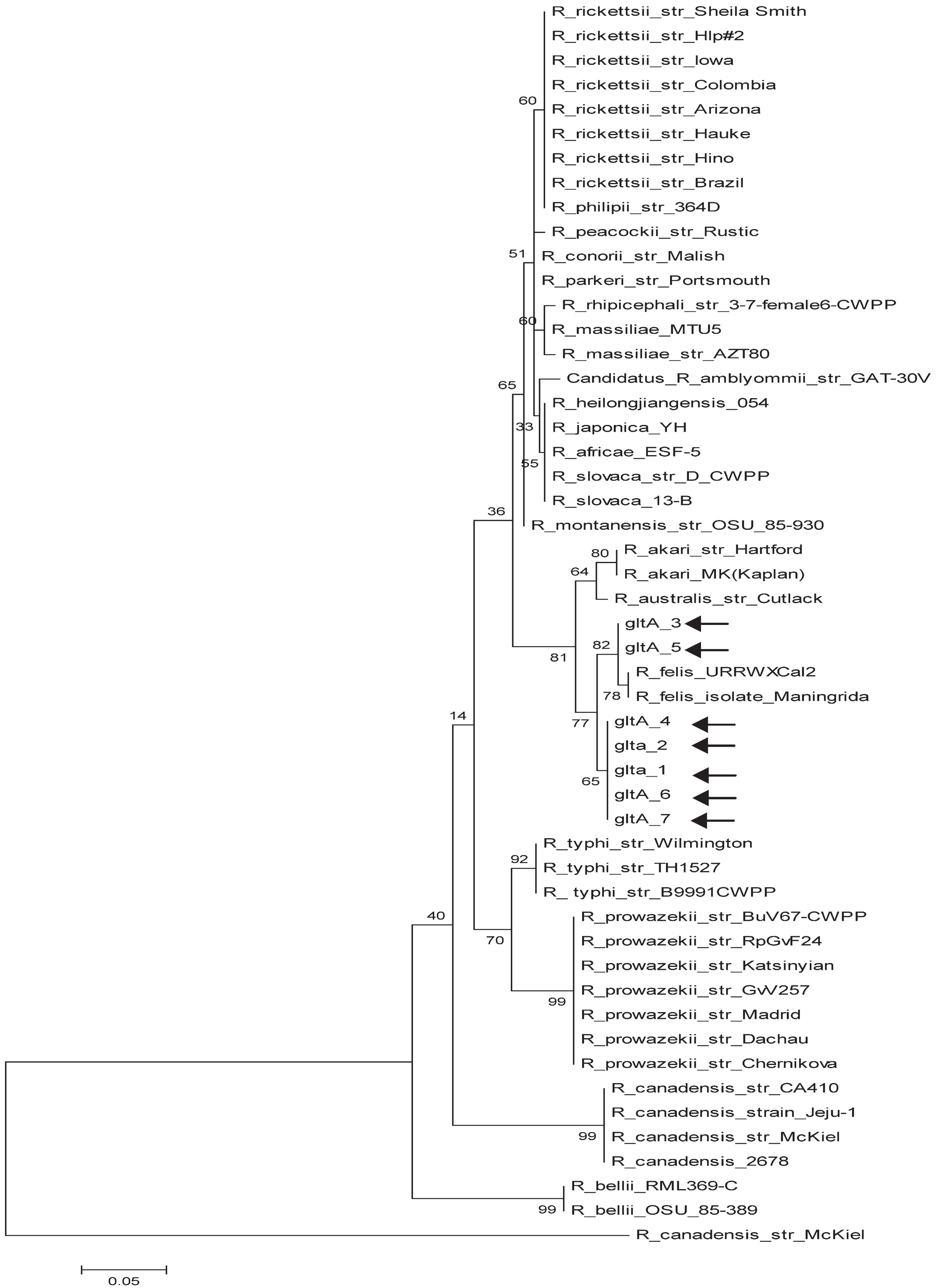

FIGURE 1. Molecular phylogenetic analysis by maximum likelihood method for the *gltA* gene (arrows point to study samples).

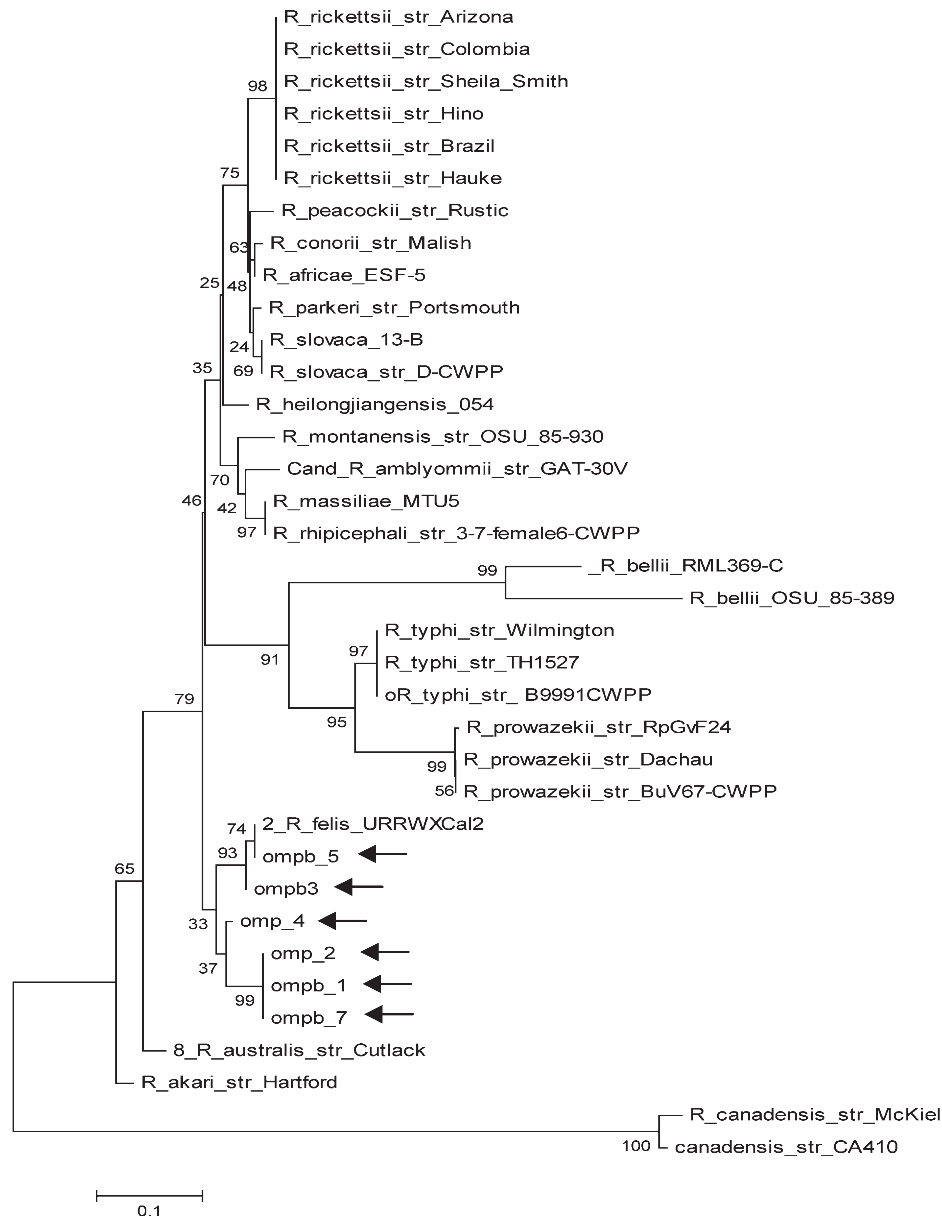

FIGURE 2. Molecular phylogenetic analysis by maximum likelihood method for the *ompB* gene (arrows point to study samples).

species with the exception of *X. cheopis*. *P. irritans* showed the highest MIR for all genes (10.0%) followed by *C. canis* (9.2%) and *C. felis* (5.3%). Additionally, the results of positive samples by flea species and host showed that *C. canis* collected from dogs was the species with the largest proportion of positive pools for *gltA* (27/37; 73%), *ompB* (26/37; 70%), and *17kD* (24/37; 65%) genes. Valid PCR reactions showed amplicons of the expected size in the positive control and no products in the negative control.

Seven pools were selected for sequencing. All of them were positive for the three genes and represented the most prevalent positive flea species in each municipality. None of the pools from La Merced met these criteria and consequently, were not included.

Sequence homology > 98% to the *R. felis* URRWXCal2 (complete genome) and other related sequences was obtained and is summarized in Table 3. Phylogenetic trees show the high homology between all sample sequences and other *R. felis* isolates in the three genes evaluated, and they confirm the monophyletic position of this species and its closeness with the *R. akari* group.

DISCUSSION

The results reported herein corroborate, for the first time in Colombia, the presence of *R. felis* in fleas collected from animals. The proportion of positive pools (for the three genes) of cat fleas (54/132; 41%) is consistent with similar studies in different countries from all continents, confirming the worldwide distribution of this emergent pathogen.[2–4] However, the MIR reported here for *C. felis* (5.3%) is lower than MIRs reported in other countries, like Brazil (14.3%),[34] Taiwan (8.2%),[35] and the United States (13.3%).[33] We must emphasize that these rates correspond to MIRs, because they were calculated with the assumption that only one flea

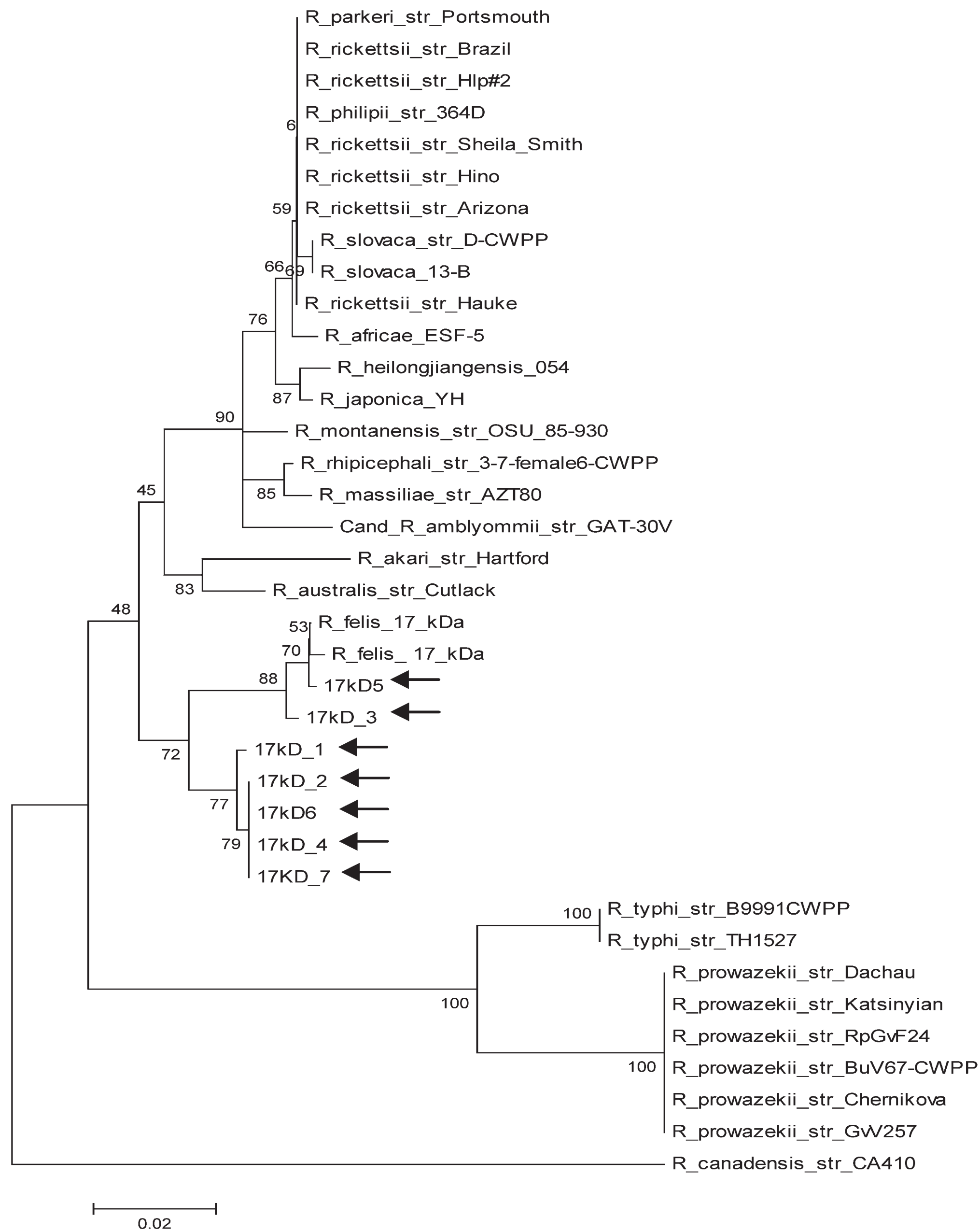

FIGURE 3. Molecular phylogenetic analysis by maximum likelihood method for the *17kD* gene (arrows point to study samples).

from each positive pool was positive for the *Rickettsia* gene analyzed; therefore, positive pools were not further corroborated to obtain the results for individual fleas, because we had limited resources.

Some studies have identified prevalence in wild-caught cat fleas ranging from 1% to 100%. Other studies found infection proportions that are similar to the proportions reported here: 22.6% in Argentina,[5] 36% in Brazil,[10] 18% in Canada,[12]

TABLE 3
Pools of fleas selected for sequencing

Sample number (identification)	Origin	Flea species	Host	Sequence homology (accession number)
1 (AP-7)	Aguadas	*C. felis*	Dog	> 98%; *R. felis* URRWXCal2 complete genome (CP000053);
2 (AZP-14)	Aranzazu	*P. irritans*	Dog	*Rickettsia* sp. cf1 and cf5 *17kDa* antigen gene (AY953286);
3 (AZP-19)	Aranzazu	*C. felis*	Cat	*Rickettsia* sp. R14 outer membrane protein B (*ompB*) gene (HM370113);
4 (PP-6)	Pácora	*C. canis*	Dog	*R. felis* clone Ar3 outer membrane protein B (*ompB*) gene (GQ385243)
5 (SP-8)	Salamina	*C. felis*	Dog	
6 (FP-31)	Filadelfia	*C. felis*	Cat	
7 (NP-16)	Neira	*C. felis*	Dog	

17.5% in France,[36] 15% in New Zealand,[37] 35% in Panama,[16] 18.8% in Taiwan,[35] and 41% in Uruguay.[23]

Other flea species with confirmed positive results for *R. felis* in this study were *C. canis* and *P. irritans*. Proportionally with *C. felis*, the sample sizes of both species were lower; however, 24 (MIR; 9.2%) and 1 (10%) samples of *C. canis* and *P. irritans* were positive for the three genes, respectively, and revealed higher MIR than *C. felis*. In the scientific literature, few reports of infection by this bacterium in these flea species have been published. In Latin America and the Caribbean, for example, only Brazil[10] and Uruguay[23] have described infection in *C. canis*, and there has been no reported infection in *P. irritans*.[37,38] For the latter flea species, we only identified published reports from the Democratic Republic of the Congo[39] and the United States.[40] Additional studies should investigate the potential role of both flea species in the epidemiology of flea-borne spotted fever in the geographical area of this study.

None of the *X. cheopis* collected in this study showed positive PCR products for *gltA*, *ompB* or *17kD* genes. However, this study was limited in the number of oriental rat fleas collected; this limitation was because of the low number of sampled rats and mice. Future research in this geographical area would increase the number of rodents (synanthropic and wild) to better understand the ecoepidemiological role of these mammals in flea-borne rickettsioses (murine typhus and *R. felis* rickettsioses).

A recent study in the same geographical area showed high seroprevalence for *R. typhi* and *R. felis* (25.2% and 17.8%, respectively).[25] The finding is in agreement with the rickettsial infection identified in fleas of the same region in this study. The absence of *R. typhi* in our sample is probably because of the very small number of *X. cheopis* (the main vector for this bacterium)[41] collected in this study. With a larger sample of *X. cheopis* and other flea species, it would be of interest to test for the presence of coinfection with *R. felis* and *R. typhi* in the same positive fleas, because it was previously reported in experimental works[42] and under natural conditions.[22,43,44]

The analysis of the sequence electropherograms showed lack of ambiguity in the nucleotide assignments for all samples included. Because the PCR products were derived from pools in which several members could be positive, the clean sequence reads suggest that the amplified fragments were highly conserved and/or that only one flea in each pool was positive for a single *Rickettsia*.

In summary, by providing evidence of appropriate arthropod vectors and their infection by *Rickettsia*, our results further corroborate the endemicity of flea-borne rickettsioses in the north of Caldas Province, Colombia.

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