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ORIGINAL ARTICLE

Comparative microbiota of *Rickettsia felis*-uninfected and -infected colonized cat fleas, *Ctenocephalides felis*

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Fleas serve as arthropod vectors for several emerging and re-emerging infectious disease causing agents including, *Rickettsia felis*. Although the prevalence of *R. felis* infection in colonies of fleas has been examined, the influence of the *R. felis* infection on flea microbiota has not been investigated. We identified three colonies of cat fleas, *Ctenocephalides felis*, with varying prevalence of *R. felis* infection (Louisiana State University (LSU), 93.8%; Professional Laboratory and Research Services Inc. (PLRS), 16.4%; Elward II (EL), 0%) and subsequently utilized polymerase chain reaction amplification, restriction fragment length polymorphism analysis and sequencing of the 1.4-kb portions of 16S rRNA genes to examine the diversity of bacteria in the flea populations. A total of 17 different bacterial 16S rRNA gene sequences were identified among the *C. felis* colonies. The prevalence of two *Wolbachia* species that were identified in each flea colony differed between colonies and *R. felis*-uninfected and -infected fleas. Species richness was unchanged among the *R. felis*-uninfected (LSU, PLRS and EL colonies) and -infected (LSU and PLRS colonies) fleas; however, between *R. felis*-uninfected and -infected fleas within both the LSU and PLRS colonies, *R. felis*-uninfected fleas have greater species richness. Diversity indices did not identify a difference in diversity between any of the flea samples. The interaction of endosymbionts within arthropods can widely impact the dissemination of vertically transmitted pathogenic bacteria; and the reciprocal may be true. These results suggest that carriage of *R. felis* has an impact on the richness of flea microbiota.

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Introduction

As important human and veterinary parasites, fleas have both a direct physical impact on their hosts and potentially transmit bacterial pathogens. For example, members of the typhus and spotted fever group *Rickettsia* cause flea-borne rickettsioses with the flea serving as the vector and reservoir. *Rickettsia typhi*, the etiological agent of murine typhus, is primarily maintained in the rat (*Rattus* spp) and rat flea (*Xenopsylla cheopis*) cycle; and a unique opossum/domestic cat and cat flea (*Ctenocephalides felis*) cycle is implicated in transmission of murine typhus in the USA (reviewed in Azad *et al.*, 1997). Spotted fever group *Rickettsia* are typically associated with ticks; however, there is increasing molecular evidence of one species, *Rickettsia felis*, in fleas including *Ctenocephalides canis* (Parola *et al.*, 2003), *Pulex irritans* (Azad *et al.*, 1997), *Anomiopsyllus nudata* (Stevenson *et al.*, 2005). Although the list of arthropods infected with *R. felis* continues to grow, *C. felis* remains the primary arthropod host (Adams *et al.*, 1990; Bouyer *et al.*, 2001; La Scola *et al.*, 2002; Pornwiroon *et al.*, 2006). While *R. felis* is maintained in an opossum-flea cycle (Azad *et al.*, 1997; Boostrom *et al.*, 2002), there is also growing evidence that domestic cats also play a role in the maintenance cycle of *R. felis* (Wedincamp and Foil, 2000; Case *et al.*, 2006). The impact of host meal source and/or composition on rickettsial transmission is not known. Another

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ecological consideration for flea-borne rickettsial diseases is the observed experimental coinfection of both *R. typhi* and *R. felis* in the same flea (Noden *et al.*, 1998), although the amount of overlap in naturally infected flea populations remains unclear (Azad *et al.*, 1997; Wiggers *et al.*, 2005).

Microbial diversity has been assessed for wild-caught *C. felis*. Utilizing culture-dependent methods, approximately 53 isolates of Gram-positive and proteobacteria from cat fleas in Australia were isolated on nutrient agar plates (Murrell *et al.*, 2003). Interestingly, there was an ectoparasite (species-specific) restriction for the genera *Nocardiopsis*, *Streptomyces* and *Flavobacterium* to *C. felis*. The isolation and subsequent sequence analysis of bacterial colonies excluded identification of obligate intracellular bacteria such as *Wolbachia* and *Rickettsia*. A culture-independent survey for *Wolbachia* in wild-caught *C. felis* collected in the United States identified *Wolbachia* in 21% of fleas assessed as determined by a nested polymerase chain reaction (PCR) and DNA sequencing of the 16S rRNA gene (Gorham *et al.*, 2003). In that study, a number of *Wolbachia* genotypes were identified with higher prevalence of *Wolbachia* infection in populations of fleas recovered from domestic animals versus wild animals.

An alternative model for assessing *C. felis*-microbe interactions utilizes colonized cat fleas. Both the original account (Adams *et al.*, 1990) and subsequent descriptions of *R. felis* (Higgins *et al.*, 1996; Bouyer *et al.*, 2001; La Scola *et al.*, 2002; Pornwiroon *et al.*, 2006) utilized colonized fleas infected with *R. felis*. Likewise, colonized cat fleas are useful for the analysis of transmission events of *R. felis* among flea populations (Wedincamp and Foil, 2000, 2002, 2003). A detailed survey of *R. felis* in eight commercial colonies in the USA provided insight into *R. felis* infection in these populations. The *R. felis* infection rates, as assessed by PCR and restriction fragment length polymorphism analysis (RFLP), varied from 43% to 93% in the colonies tested. Interestingly, many of these colonies were either initiated or replenished with fleas from a source that was *R. felis*-infected (Higgins *et al.*, 1994).

The influence of vertically transmitted bacteria (for example, *Wolbachia* and *Spiroplasma*) on reproduction has been described for a number of arthropods (reviewed in Bandi *et al.*, 2001), possibly having a broad impact on vertically transmitted rickettsial pathogens. Sex ratio distortion (SRD), favoring female progeny as a continual means for vertical transmission of microorganisms, occurs through a variety of mechanisms including male killing, feminization and parthenogenesis. Additionally, cytoplasmic incompatibility (CI) is an alternative method for reproductive manipulation favoring vertical transmission of microorganisms. SRD has not been identified in fleas; however, either in concert with other microorganisms or alone, *Rickettsia* and *Wolbachia* could potentially manipulate reproductive activity.

Despite the prevalence of *R. felis* and endosymbionts (primarily *Wolbachia*) in numerous flea colonies and wild-caught fleas, respectively, the interplay between these genera in individual fleas has not been examined. Likewise, previous studies have not accounted for the contribution of the host blood meal source and the nutritional status of the fleas, which has been documented to influence bacterial communities in arthropods (Moreno *et al.*, 2006). As a result of the high level of prevalence and stable vertical transmission of *R. felis* in some colonies of *C. felis*, we hypothesize that *R. felis* directly influences the prevalence of other species of bacteria within the flea. To assess the interplay between endosymbionts and the flea host and the potential impact of infections on vector physiology, we must define the microbial profile before assessing the impact of *R. felis* on the flea host. Toward this objective, we utilized the molecular analysis of the 16S rRNA gene sequence to define the microbiota of three colonies of cat fleas with different blood meal sources and varying levels of *R. felis* infection in the flea population.

Materials and methods

Source of fleas

Newly emerged, unfed adult *C. felis* were either obtained from the Louisiana State University (LSU) colony or purchased from commercial vendors. The LSU adult fleas are maintained on domestic shorthair cats at the School of Veterinary Medicine in a manner previously described by Henderson and Foil (1993). A second group of fleas was purchased from Professional Laboratory and Research Services (PLRS) Inc. (Corapeake, NC, USA). This colony is maintained on cats. A third group of fleas was purchased from Elward II (EL) (Soquel, CA, USA). This colony is maintained on sheep blood via an artificial feeding system (Wade and Georgi, 1988).

DNA isolation

Fleas were washed five times in 70% ethanol, followed by three washes with DNase–RNase-free water. Fleas were blotted dry and transferred individually to 1.5 ml microcentrifuge tubes containing 20 μl of DNase–RNase-free water. Samples were ground with plastic pestles and heated at 95°C for 5 min. After brief centrifugation to collect contents, 2.5 μl of lysates were used as PCR templates. For comparison of DNA isolation techniques, DNA was isolated from three individual *R. felis*-infected fleas from the LSU colony using the QIAGEN DNeasy tissue kit as described previously (Henry *et al.*, 2007). All DNA samples were utilized as described below for PCR amplification and RFLP analysis and no differences between DNA isolation

techniques or pooled versus individual flea analyses were identified with respect to total bacterial species present (data not shown).

PCR assessment of rickettsial infection
Flea lysates from each group were assessed for rickettsial infection using a nested PCR. Two sets of oligonucleotide primers (Integrated DNA Technologies Inc., Coralville, IA, USA) for *Rickettsia* genus-specific 17-kDa antigen gene, *Rr*17.61p-*Rr*17.492n (Williams *et al*., 1992) and *Rc*17.159p-*Rc*17.308n (5′-GCTTGTTGGAGTAGGTGTAGGTG-3′ and 5′-CGCCATTCTACGTTACTACCAC-3′), which generate a 434 bp product and subsequently a 150 bp product, the internal nested region of this gene, were used for PCR amplification. PCR products were amplified using PCR Master Mix (Promega, Madison, WI, USA) with the cycling parameters of an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 45 s and a final extension at 72°C for 7 min. Genomic DNA from *R. montanensis* M5/6 (positive control for 17-kDa antigen gene) and water (negative control) were run to ensure proper amplification and lack of contamination, respectively. Amplified products were visualized on ethidium bromide-stained 1.5–2% agarose gels. For each colony of flea, the 17-kDa antigen gene amplicons were sequenced by the dye terminator method on a 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA) at School of Veterinary Medicine, LSU.

Amplification and cloning of bacterial 16S rRNA genes
The 1.4-kb partial sequence of the 16S rRNA gene was amplified from individual *R. felis*-uninfected or -infected flea lysate using PCR Master Mix (Promega) together with universal bacterial primers fD1 and rP1–3 (Weisburg *et al*., 1991). The condition used was as follows: 94°C for 3 min, 35 cycles of 94°C for 30 s, 52°C for 45 s, 72°C for 2 min and 72°C for 7 min. 16S rRNA gene products were pooled (flea colony/no. of pools/no. of fleas per pool) for each group of *R. felis*-uninfected (LSU/1/2; PLRS/2/8; EL/2/8) or -infected fleas (LSU/2/8; PLRS/1/8), gel-purified using Wizard SV Gel Clean-Up System (Promega) and ligated into pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

RFLP analysis
Aliquots of 16S rRNA gene PCR products amplified individually from up to 40 clones from each ligation pool by the fD1 and rP1–3 primer set were digested with 4 U of restriction enzyme *Rsa*I and *Alu*I (New England Biolabs, Ipswich, MA, USA) at 37°C for 3 h in the manufacturer's recommended reaction buffer. Digests were visualized on ethidium bromide-stained 2.5% agarose gels.

DNA sequencing and analysis
At least three clones for each RFLP pattern were sequenced as described above. The primers used for sequencing a portion of the 16S rRNA gene were M13F, M13R and the internal primer, 16S rRNA-walk (5′-AGGGTTGCGY[C + T]TCGTTGCK[G + T]-GR[A + G]AC-3′). Sequence contigs were assembled using Vector NTI software (Invitrogen) and subjected to BLASTn analysis (http://www.ncbi.nlm.nih.gov/blast) for similarity to known sequences. All unique sequences of 16S rRNA gene were deposited in the GenBank database under accession numbers EF121340 to EF121353.

Molecular phylogenetic analysis
A multiple sequence alignment for *C. felis*-associated bacterial 16S rRNA gene sequences, closest database match and most similar sequences for known species was conducted with the sequence alignment for Clustal W. The resulting alignment was used for generation of neighbor-joining and maximum-parsimony gene trees using the Molecular Evolutionary Genetics Analysis (MEGA) version 3.1 software (Kumar *et al*., 2004). Phylogenetic tree construction utilized Kimura 2-parameter distances. A bootstrap resampling strategy with 500 replicates was employed to assess reliability of the trees.

Statistical analysis
Differences in microbial communities in fleas were assessed in terms of prevalence of common bacteria (clones) within each colony and between *R. felis*-uninfected and -infected cohorts of each colony using Fisher's exact test with a probability less than 0.05 considered a significant difference. Rarefaction was used to compare species richness (number of species) among and between *R. felis*-uninfected and -infected samples. Accumulating curves were generated by selecting 100 random samplings, with replacement, for each sample using EstimateS 7.5 (http:purl.ock.org/estimates), with differences at the 95% confidence interval level being considered significant. Observed species richness was plotted as a function of the number of clones utilizing the sample-based Mao Tau method (Colwell *et al*., 2004). The rare-isolate-based Chao1 was used to estimate the total number of bacterial species (Chao, 1984).

Species diversity (number of different species in a cohort of fleas) among the samples was assessed using Fisher's, Shannon's and Simpson's (Simpson and 1/Simpson) diversity indices.

Results

PCR assessment of rickettsial infection
To investigate the effect of the carriage of *R. felis* on flea microbiota, rickettsial infection was assessed in three colonies of *C. felis*. A total of 32 (LSU colony),

55 (PLRS colony) and 50 (EL colony) cat fleas were tested by PCR amplification with the *Rr*17.61p-*Rr*17.492n primer set and 87.5% (28/32), 0% (0/55) and 0% (0/50), respectively, were found to be positive. The percentage of infection increased to 93.8% (30/32), 16.4% (9/55) and 0% (0/50) in the corresponding samples using a subsequent nested PCR with the *Rc*17.159p-*Rc*17.308p primer set, indicating high, moderate and no levels of rickettsial infection for LSU, PLRS and EL colonies, respectively. The DNA amplicons of 434 bp (LSU only) and 150 bp (LSU and PLRS) were cloned and sequenced to confirm the specificity of the PCR test; the nucleotide sequences of both amplicons were 100% matched to sequences reported for *R. felis* in the GenBank database (accession numbers CP000053 and AF195118).

Comparison of bacterial species in C. felis *with varying levels of rickettsial infection*

The near full-length sequences of the 16S rRNA genes were individually PCR-amplified from randomly selected *R. felis*-uninfected and -infected *C. felis* samples. Pools of two (*R. felis*-uninfected LSU fleas only) or eight (all other flea samples) DNA amplicons from flea samples with varying levels of rickettsial infection in each colony were cloned; the 16S rRNA gene PCR products amplified individually from the positive clones were digested by *Rsa*I and *Alu*I. Analysis of 224 clones identified 17 distinct RFLP patterns, indicating that both *Rsa*I and *Alu*I were needed for accurate differentiation of the clones because the individual enzymes generated a lower number of different operational taxonomic units (Figures 1a and b).

DNA sequences obtained from representatives of each RFLP pattern were compared with known sequences in the GenBank database. RFLP patterns and the corresponding most closely matched bacterial species found in *R. felis*-uninfected and -infected fleas from each colony are shown in Table 1. A total of 12 bacterial species related to the genera *Acidovorax*, *Acinetobacter*, *Bergeyella*, *Bosea*, *Klebsiella*, *Methylobacillus, Rickettsia, Spiroplasma*, *Stenotrophomonas*, *Staphylococcus* and *Wolbachia* were detected; and, an additional five species were closest matched to various uncultured bacteria. Molecular phylogenetic analysis supports the characterization of 16S rRNA gene sequences of *C. felis*-associated bacteria listed in Table 1 as closely related, sometimes identical, to sequences obtained from BLASTn analysis (Figure 2). *Wolbachia pipientis* from *C. felis* (99% identity to GenBank accession number AJ628416) and the uncultured ant-associated bacterium (GenBank accession number DQ068830), which groups with *Wolbachia* under phylogenetic analysis were identified in all of the flea colonies. Significant differences in the prevalence of *W. pipientis* were observed between each colony with the highest prevalence in the EL fleas that were observed to be free of *R. felis* infection by nested PCR with oligonucleotide primers for *Rickettsia* genus-specific 17-kDa antigen gene. The *R. felis*-uninfected LSU fleas had significantly higher prevalence of *Wolbachia*

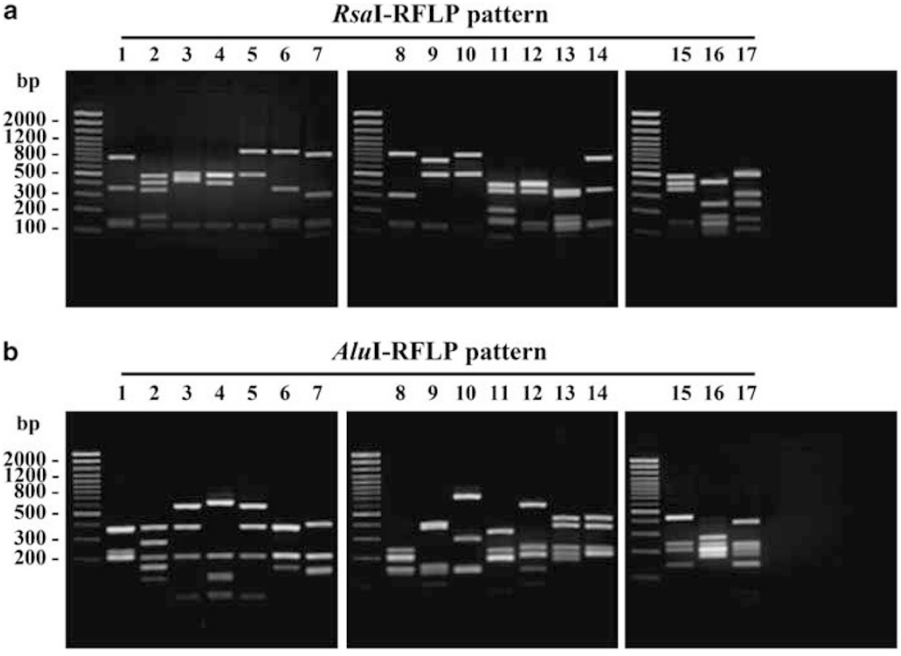

Figure 1 Total 224 positive 16S rRNA clones obtained from *Ctenocephalides felis* samples with varying levels of *Rickettsia felis* infection were individually PCR-amplified and the 16S rRNA gene PCR products (~1.4 kb) were digested with *Rsa*I (**a**), *Alu*I (**b**). Seventeen distinct restriction fragment length polymorphism (RFLP) patterns were identified. Marker (a 100-bp ladder) sizes are listed to the left of the gel.

Table 1 Bacteria identified in *Rickettsia felis*-uninfected and -infected *Ctenocephalides felis* were differentiated by RFLP analysis and putative identification was assigned based on the closest matched 16S rRNA gene sequence in the GenBank database

	Flea colony	*RFLP pattern*[a]	*Length (bp)*	*Closest BLASTn match (accession no.)*	*No. identical/total (% identity)*	*Prevalence in clones analyzed (from no. of pool)*	*Accession no.*[b]
PLRS	Uninfected	1	1459	Uncultured bacterium (DQ321554)	1458/1460 (99)	9/59 (2)	EF121340
		2	1467	*Stenotrophomonas* sp (DQ256392)	1466/1467 (99)	32/59 (2)	EF121341
		3	1464	Uncultured bacterium (AF423229)	1460/1465 (99)	6/59 (2)	EF121342
		4	1475	*Staphylococcus saprophyticus* (AP008934)	1475/1475 (100)	4/59 (2)	—
		5	1462	*Klebsiella pneumoniae* (X93214)	1451/1458 (99)	3/59 (2)	EF121343
		6	1460	Uncultured *Acinetobacter* sp (DQ366106)	1458/1460 (99)	5/59 (2)	EF121344
	R. felis-infected	2	1467	*Stenotrophomonas* sp (DQ256392)	1466/1467 (99)	24/37 (1)	—
		4	1475	*Staphylococcus saprophyticus* (AP008934)	1475/1475 (100)	3/37 (1)	—
		7	1425	*Wolbachia pipientis* (AJ628416)	1387/1391 (99)	5/37 (1)	EF121345
		8	1426	Uncultured bacterium (DQ068830)	1404/1426 (98)	2/37 (1)	EF121347
		10	1444	*Spiroplasma* sp (AM087471)	1437/1444 (99)	3/37 (1)	EF121346
EL	Uninfected	4	1475	*Staphylococcus saprophyticus* (AP008934)	1475/1475 (100)	3/39 (2)	—
		7	1425	*Wolbachia pipientis* (AJ628416)	1387/1391 (99)	25/39 (2)	—
		8	1426	Uncultured bacterium (DQ068830)	1404/1426 (98)	11/39 (2)	—
LSU	Uninfected	7	1425	*Wolbachia pipientis* (AJ628416)	1387/1391 (99)	3/41 (1)	—
		8	1426	Uncultured bacterium (DQ068830)	1404/1426 (98)	27/41 (1)	—
		11	1439	*Bergeyella* sp (AJ575430)	1425/1429 (99)	3/41 (1)	EF121348
		12	1451	*Acidovorax* sp (AF458096)	1449/1451 (99)	3/41 (1)	EF121349
		13	1460	Uncultured bacterium (AF314418)	1451/1459 (99)	1/41 (1)	EF121350
		14	1459	*Acinetobacter junii* (AB101444)	1459/1459 (100)	1/41 (1)	—
		15	1465	*Methylobacillus flagellatus* (CP000284)	1448/1465 (98)	1/41 (1)	EF121351
		16	1453	Uncultured Burkholderiales bacterium (DQ234135)	1445/1453 (99)	1/41 (1)	EF121352
		17	1408	*Bosea thiooxidans* (AF508112)	1332/1406 (94)	1/41 (1)	EF121353
	R. felis-infected	7	1425	*Wolbachia pipientis* (AJ628416)	1387/1391 (99)	11/48 (2)	—
		8	1426	Uncultured bacterium (DQ068830)	1404/1426 (98)	2/48 (2)	—
		9	1421	*Rickettsia felis* URRWXCal2 (CP000053)	1421/1421 (100)	35/48 (2)	—

Abbreviations: LSU, Louisiana State University; PLRS, Professional Laboratory and Research Services Inc.; EL, Elward II; RFLP, restriction fragment length polymorphism.
[a]RFLP patterns correspond to Figures 1a and b.
[b]Unique 16S rRNA gene sequences identified, corresponding to each RFLP pattern, were deposited into the GenBank database.

sp (GenBank accession number DQ068830) when compared to other flea samples. *Staphylococcus saprophyticus* was identified in equal prevalence in clones assessed (~7%) from the EL samples and the *R. felis*-uninfected and -infected PLRS samples. In the PLRS colony, a total of nine unique 16S rRNA gene sequences were identified in two pools of *R. felis*-uninfected and one pool of *R. felis*-infected samples with the predominant 16S rRNA clone identified as *Stenotrophomonas* sp (32/59 clones for *R. felis*-uninfected and 24/37 clones for the *R. felis*-infected). Other insect-associated bacteria were putatively identified in the PLRS *R. felis*-free samples including *Acinetobacter* and *Klebsiella* species. The *R. felis*-infected samples lacked these species, but did have a *Spiroplasma* sp detected in 3/37 clones assessed. Also, despite the positive amplicon by PCR with *Rickettsia* genus-specific17-kDa antigen gene primers, the *R. felis* 16S rRNA gene was not detected by this method (0/37) in the clones assessed. Combined with the required nested PCR for amplification of the *Rickettsia* genus-specific 17-kDa antigen gene, these results indicate a low level of *R. felis* infection in these samples.

The predominantly identified clone (35/48) in the *R. felis*-infected samples from the LSU colony was *R. felis*. Both *Wolbachia* spp were identified and constituted the remainder of clones. For the *R. felis*-uninfected LSU sample, DNA amplicons from the two fleas were pooled and nine different clones were identified with the uncultured *Wolbachia* sp (GenBank accession number DQ068830) as the predominant clone (27/41). *Wolbachia pipientis*, *Acidovorax* and *Bergeyella* were each identified in 3/41 clones assessed. Five of the nine identified sequences closely related to *Acinetobacter*, *Bosea*, *Methylobacillus* and uncultured bacterium were each identified in 1/41 clones assessed.

In most of the samples, the number of clones was enough to capture the species richness; rarefaction demonstrated sample observations leveled off and did not significantly differ from estimated total species richness. With the exception of the *R. felis*-uninfected LSU fleas, species richness accumulation curves indicate that estimated species and observed species were comparable for *R. felis*-uninfected and -infected fleas from each colony. Species richness did not differ in either *R. felis*-uninfected or -infected fleas between the three colonies (LSU, PLRS and EL); therefore, the source of the blood meal did not influence species richness. *R. felis* infection within a colony did influence

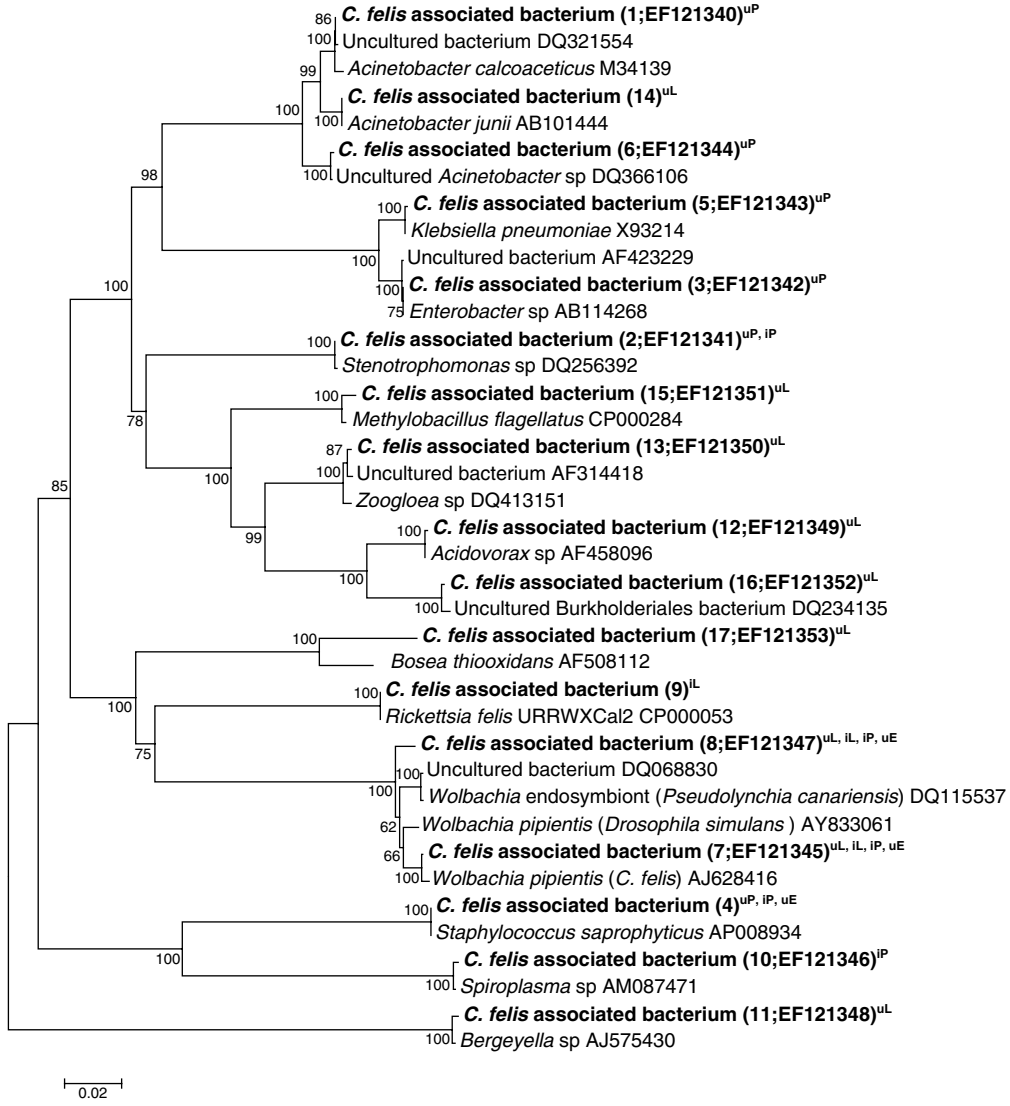

Figure 2 Molecular phylogenetic analysis of *Ctenocephalides felis*-associated bacteria (RFLP pattern #; corresponding GenBank accession number) based on 16S rRNA gene sequences identified in *Rickettsia felis*-uninfected (u) and -infected (i) fleas from the LSU (L), PLRS (P) and EL (E) flea colonies and 16S rRNA gene sequences for the closest match and most similar cultured bacteria. Aligned nucleotide sites were subjected to analysis using the MEGA version 3.1 software. A neighbor-joining tree is shown; maximum parsimony produced a similar topography. The bootstrap values obtained with 500 resamplings are presented in the nodes. LSU, Louisiana State University; PLRS, Professional Laboratory and Research Services Inc.; EL, Elward II; RFLP, restriction fragment length polymorphism.

species richness; decreased richness was observed in *R. felis*-infected fleas when compared to *R. felis*-uninfected fleas within the LSU and PLRS colonies. Although species richness was significantly reduced by the presence of *R. felis* in a colony, the diversity of bacteria present in the colony was not affected, when analyzed with Shannon's and Simpson's diversity indices. The Fisher Alpha index did indicate a greater diversity in *R. felis*-uninfected LSU fleas when compared to *R. felis*-infected fleas.

Discussion

Despite the recognition of *R. felis* as an emerging pathogen and the cosmopolitan distribution of both the pathogen and *C. felis*, the influence of the *R. felis* infection on the arthropod host has not been examined. The interaction between individual bacterial species within the vector may provide insight into the role of endosymbiont and pathogenic bacteria in flea fitness and define vectorial capacity of the flea population. Our long-term objective is to determine the influence of *Rickettsia* on the biological parameters of the flea life cycle; therefore, to first define the microbial profile, we surveyed the microbiota of *C. felis* infected with *R. felis* then compared the results to *C. felis* lacking *R. felis* infection.

In the current study, cloned 16S rRNA amplicons were subjected to PCR/RFLP, sequencing analysis, and subsequently utilized to compare the diversity

of bacteria in three colonies of *C. felis* with varying levels of *R. felis* infection in the flea populations. PCR/RFLP is a simple and reproducible method of bacterial differentiation that does not require special equipment and is performed in many laboratories (Shima *et al.*, 2004; Ciantar *et al.*, 2005). Moreover, by using PCR the limitations of traditional culture-dependent technique, which requires the isolation and culture of bacteria, is overcome (Ranjard *et al.*, 2000). The widespread use of PCR/RFLP for the characterization of *Rickettsia* (Regnery *et al.*, 1991; Gage *et al.*, 1994; Roux *et al.*, 1996) including *R. felis* (Azad *et al.*, 1992; Schriefer *et al.*, 1994; Boostrom *et al.*, 2002) has facilitated rickettsial identification in the absence of culture.

The use of colonized *C. felis* greatly advances research efforts toward the delineation of flea biology, control and pathogen interaction (Thomas *et al.*, 2004). Previous reports identified the presence of *R. felis* (called ELB agent) in eight commercial *C. felis* colonies assessed (Higgins *et al.*, 1994). Three of the colonies assessed by Higgins *et al.* (1994) were re-examined in the current study. Since the first study was conducted, the prevalence of *R. felis* infection in colonies from California (EL) and North Carolina (PLRS) decreased from 83% to 0% and from 50% to 16.4%, respectively, and increased in the LSU colony from 86% to 93.8%. Located at different sites, the three colonies (two of which are maintained for commercial purposes) vary in rearing specifications; therefore, it is only possible to speculate what facilitated the changes in prevalence of *R. felis* within the flea populations over time. For example, the LSU colony has been maintained for approximately 3 years without the infusion of outside fleas and within the last 2 years it has experienced a severe population loss followed by a rapid expansion in the population. The high prevalence of *R. felis* in the fleas indicates a relationship between survival and *R. felis* infection.

Murrell *et al.* (2003) examined the culture-dependent microbial profiles of wild-caught *C. felis* and identified high levels of diversity within the bacterial community, not present in the colonized *C. felis*. However, there were highly ubiquitous species of bacteria identified in colonized and wild-caught *C. felis* assessed in the current study and previous reports (Gorham *et al.*, 2003; Murrell *et al.*, 2003). *S. saprophyticus*, which has been described during microbial surveys of fleas, lice and ticks (Murrell *et al.*, 2003), was identified in equal prevalence in the EL samples and the PLRS samples (*R. felis*-uninfected and -infected). Two species of bacteria, *Wolbachia pipientis* from *C. felis* and an uncultured *Wolbachia*, are present in all three flea colonies. Comparisons of our 16S rRNA gene sequences (Genbank accession numbers EF121345 (1425 bp) and EF121347 (1426 bp)) to the published sequence of *Wolbachia* endosymbiont from wild-caught *C. felis* (493 bp) (GenBank accession number AY157503; (Gorham *et al.*, 2003)) exhibited 12 nucleotide differences with varying positions. However, there were 32 nucleotide differences between our two 16S rRNA gene sequences (GenBank accession numbers EF121345 and EF121347).

The contribution of the host blood meal source was compared using a flea colony that is maintained on sheep blood via an artificial feeding system versus a live host (cat) fed fleas. In the current study, we utilized unfed adult fleas to reduce the variability of bacterial infections associated with blood feeding during the adult stage, which has been described for other blood feeding arthropods (Moreno *et al.*, 2006). Neither species richness nor diversity was impacted as a result of colony maintenance on an artificial versus a live host. The lack of significant difference is likely due to the limited differences in larval rearing techniques. However, as with other holometabolous insects, the contribution of the larval diet must be considered (Pidiyar *et al.*, 2004) a source of bacteria in the midgut and, hence, the environmental bacteria identified in this study.

In *R. felis*-infected samples from both the PLRS and LSU colonies, limited richness was associated with rickettsial infection. This is noteworthy because unique insect-associated bacteria were identified in the PLRS *R. felis*-free samples, including *Acinetobacter* and *Klebsiella* species. The *R. felis*-infected samples lacked these species, but did have a *Spiroplasma* sp detected in approximately 8% of clones. Also, despite the positive amplicon by diagnostic PCR, *R. felis* 16S rRNA gene was not detected by this method. The pronounced difference between the prevalence of *R. felis* in flea populations would imply that a pre-existing infection with a vertically transmitted *R. felis* limited microbial richness, but not diversity.

The nutritional status of the arthropod in relation to bacterial composition has been examined for other blood feeding arthropods (Moreno *et al.*, 2006). There was a correlation between life stage, feeding and prevalence of some species of bacteria (for example, *Borrelia* and *Sphingobacteriaceae*) in *Ixodes scapularis* ticks. In the wild-caught flea surveys, most samples were removed from vertebrate hosts, most likely after a blood meal. The microbial profile of the flea in the current study is reflective of the true nature of unfed fleas acquiring bacteria during both transovarial transmission and larval feeding and maintaining them during the molt. Therefore, differences in microflora carried through trans-stadial transmission indicates that *R. felis* influences microbial profiles in immature and adult fleas. Indeed, we observed decreased species richness in *R. felis*-infected fleas in two separate colonies of fleas. Whether *R. felis* infection negatively impacts flea fitness or enhances survival via blocking harmful infections is not examined in this study, but the idea is intriguing. The tissue distribution, if unique for the different species, including

R. felis, will provide insight into the dynamic microbial interactions within the flea.

The influence of vertically maintained bacteria on reproduction mechanisms of arthropods has been described for a number of bacteria–host associations. Specifically, the contribution of *Wolbachia* to reproductive manipulation and interspecific interaction between *Wolbachia* and other symbionts (Goto *et al.*, 2006) is well documented. Parthenogenesis has been reported not to occur in fleas (Zakson-Aiken *et al.*, 1996); however, the contribution of bacteria to reproduction through other mechanisms such as CI has not been examined. The current study has identified the same two *Wolbachia* species, as determined by 16S rRNA gene sequences, in each of the colonies assessed. Remarkably, both *Wolbachia* were the predominate bacteria in the EL fleas, 64% and 28% of clones assessed, respectively, that were observed to be free of *R. felis* infection by diagnostic PCR. Conversely, in the LSU colony with a high prevalence of *R. felis*-infection, these same bacteria (at a ratio of 2:1 in EL) are identified at a ratio of 5:1 with a large decrease in the uncultured *Wolbachia* (GenBank accession number DQ068830). The ability of *Spiroplasma* to regulate *Wolbachia* load and distribution within arthropods has been described (Goto *et al.*, 2006). Whether or not rickettsiae negatively impact other endosymbionts in the fleas has not been determined.

In summary, PCR/RFLP and sequence analysis has been used to characterize microbial profiles and the influence of *R. felis* on these microbial communities of cat fleas. Distinction between PCR-amplified 16S rRNA cloned sequences by RFLP was effective, but required at least two enzymes for definitive separation. The significance of this emerging pathogen, combined with its close association with the arthropod host, make this a worthy model to examine. Specifically, the contribution of *R. felis* to flea fitness and reproductive behavior, and the interacting factors associated with the prevalent *Wolbachia* infection, should be examined further as this may directly influence rickettsial transmission to vertebrate hosts.

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