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Rickettsia felis: molecular characterization of a new member of the spotted fever group

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In this report, placement of *Rickettsia felis* in the spotted fever group (SFG) rather than the typhus group (TG) of *Rickettsia* is proposed. The organism, which was first observed in cat fleas (*Ctenocephalides felis*) by electron microscopy, has not yet been reported to have been cultivated reproducibly, thereby limiting the standard rickettsial typing by serological means. To overcome this challenge, several genes were selected as targets to be utilized for the classification of *R. felis*. DNA from cat fleas naturally infected with *R. felis* was amplified by PCR utilizing primer sets specific for the 190 kDa surface antigen (rOmpA) and 17 kDa antigen genes. The entire 5513 bp *rompA* gene was sequenced, characterized and found to have several unique features when compared to the *rompA* genes of other *Rickettsia*. Phylogenetic analysis of the partial sequence of the 17 kDa antigen gene indicated that *R. felis* is less divergent from the SFG rickettsiae than from the TG rickettsiae. The data corroborate results from previous reports that analysed the citrate synthase, 16S rRNA, *rompB* (135 kDa surface antigen), *metK*, *ftsY*, *polA* and *dnaE* genes that placed *R. felis* as a member of the SFG. The organism is passed transstadially and transovarially, and infection in the cat flea has been observed in the midgut, tracheal matrix, muscle, hypodermis, ovaries and testes.

Keywords: *Rickettsia felis*, rickettsial outer-membrane protein A, 17 kDa gene, *Ctenocephalides felis*, tandem repeat domain

INTRODUCTION

In 1990, during a study investigating potential vectors for *Ehrlichia risticii*, rickettsia-like organisms were observed in the midgut epithelial cells of adult cat fleas, *Ctenocephalides felis*, by electron microscopy (Adams *et al*., 1990). The organisms were found only in a group of fleas obtained from El Labs, from which the original designation of the organism (ELB) was derived, and not in any of the other three sources of fleas. The organisms were described as having an ultrastructure and infection pattern similar to that of *Rickettsia typhi*. The organisms were 0·25–0·45 μm in diameter by 1·5 μm in length and were found not only in the midgut, but also in the tracheal matrix, muscles and reproductive tissues of the fleas. The organisms contained trilaminar cell walls that were characteristic of rickettsiae with a well-defined inner cell membrane and outer membrane. Measurements of the microcapsular layer, outer and inner leaflets of the outer membrane, and the periplasmic space strongly resembled those in other *Rickettsia* species. The first attempts to characterize the organism involved the amplification of the 17 kDa antigen, citrate synthase (CS) and 190 kDa antigen (*rompA*) genes (Azad *et al*., 1992). The ELB

Abbreviations: CS, citrate synthase; SFG, spotted fever group; TG, typhus group.

The GenBank accession numbers for the 17 kDa protein gene and *rompA* sequences of *Rickettsia felis* are AF195118 and AF191026, respectively.

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agent was found to be distinguishable from *R. typhi* by RFLP analysis of the 17 kDa gene product digested with *Aha*II or *Alu*I. The CS gene was used to confirm that the organism found in the cat fleas was the ELB agent and not *R. typhi*, which can also be found occasionally in these fleas. The RFLP pattern of the CS gene amplified from the ELB agent in cat fleas differed from that of *R. typhi*. Attempts at that time to amplify the *rompA* gene from the ELB agent proved to be unsuccessful. It has also been shown by two experimental observations that the ELB agent can be transmitted trans-stadially and transovarially. Unfed cat fleas that were negative by PCR for the ELB agent tested positive for the 17 kDa protein gene of the ELB agent after feeding on infected cats (Azad *et al*., 1992). Also, the ELB agent was present in freshly deposited eggs as determined by PCR (Azad *et al*., 1992). The first serological assays for the ELB agent were also conducted in this study. Antisera and mAbs generated against *R. typhi* were used to examine smears of newly emerged fleas from both the El Labs and negative controls. Indirect immunofluorescent staining detected the ELB agent in the sample fleas, but not in the control fleas. In surveys of fleas in Los Angeles County, CA and in Texas, the 17 kDa and CS genes were used to investigate the natural occurrence of the ELB agent (Williams *et al*., 1992; Schriefer *et al*., 1994a). The results from both studies indicated that infection of the cat flea with the ELB agent is more prevalent than with *R. typhi*. One study reported an infection rate of 3·8 % for the ELB agent (Schriefer *et al*., 1994a). This investigation also detected the ELB rickettsia within the spleen of opossums, thus suggesting that this animal might play a role in the maintenance of the ELB agent in nature and proving the capacity of the rickettsia to cause disseminated infection. The ELB agent has been identified in flea colonies from various regions of the United States through the use of RFLP analysis of PCR products of the 17 kDa and CS genes (Higgins *et al*., 1996). Analysis of the eight colonies showed that they were infected with the ELB agent with a range of prevalence within each colony of 43–93 %. The possible source of ELB in these colonies was subsequently traced to the El Labs, which provided fleas as starter stock or to replenish the colony. Attempts to infect mammalian cells and SCID mice with the ELB agent were not successful. Two publications are based upon the study of organisms considered to represent ELB agent propagated in cell culture (Radulovic *et al*., 1995a, b); however, the cultured agent could not be reproducibly propagated and maintained in further culture. In addition, there is the possibility of contamination of the later passages of the culture with *R. typhi*, which has also been found to infect the cat flea. Data from these two reports are not included in the evidence included here that the ELB agent is a novel *Rickettsia* species. In 1996, it was proposed that the ELB agent be designated *Rickettsia felis* in recognition of its discovery and origin in the cat flea (Higgins *et al*., 1996). Subsequent additions to our knowledge of *R. felis* have used this name in the biomedical and scientific literature (Noden *et al*., 1998; Andersson & Andersson, 1999; Andersson *et al*., 1999; Bouyer *et al*., 1999).

There have also been reports implicating the involvement of *R. felis* in human disease, indicating its potential importance as a newly emerging pathogen (Schriefer *et al*., 1994a; Zavala-Velazquez *et al*., 2000). Since *R. felis* has thus far resisted attempts at cultivation, thereby limiting the standard rickettsial typing by serological means, the organism was further characterized through the utilization of molecular means as has been employed to describe other uncultivated rickettsial organisms (Anderson *et al*., 1992; Neibylski *et al*., 1997). The *rompA* gene from cat fleas containing *R. felis* was amplified by PCR using different primers and amplification conditions and sequencing data for the 17 kDa protein gene were obtained. The *rompA* gene was selected as one of our target genes based on the fact that only spotted fever group (SFG) rickettsiae contain this gene and that the proof of the existence of this gene in *R. felis* would have a significant impact upon its classification (Roux *et al*., 1996). In this study, the successful sequencing and characterization of the *rompA* gene of *R. felis* is reported. These data, along with other sequence information on the CS, *rompB*, 17 kDa protein, *metK*, *ftsY*, *polA* and *dnaE* genes, are the justification for describing *R. felis* as a member of the SFG.

METHODS

Source of infected fleas. The infected cat fleas were maintained in the laboratory colony of the Department of Entomology, Louisiana State University Agricultural Center (Henderson & Foil, 1993).

DNA isolation. A pool of infected cat fleas was washed with sterile PBS (137 mM sodium chloride, 2·7 mM potassium chloride, 4·3 mM dibasic sodium phosphate, 1·4 mM potassium phosphate) and homogenized. The homogenate was resuspended in 1 ml PBS containing 1 % SDS and 1 % proteinase K, placed in a 1·5 ml microcentrifuge tube and incubated for 1 h at 37 °C followed by a 10 min incubation at 56 °C. A series of phenol/chloroform extractions was performed on the homogenate followed by a single chloroform extraction. The DNA was ethanol-precipitated and dried, and the pellet was resuspended in sterile water.

PCR. Amplification of an aliquot of the DNA extracted from fleas using the outer primers for the *Rickettsia* 17 kDa gene was performed as described previously (Webb *et al*., 1990; Schriefer *et al*., 1994b; Higgins *et al*., 1996). Six micrograms of DNA in a 5 µl volume of DNA extract was added to 1 × PCR buffer (Boehringer Mannheim), 200 µM nucleotide mixture, 10 pmol each primer, 1·25 units *Taq* polymerase (Boehringer Mannheim) and distilled water to a final volume of 100 µl. The PCR was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus). The PCR product from this reaction was further amplified using a set of nested primers for the 17 kDa gene (Higgins *et al*., 1996). PCR conditions for both reactions were as previously described (Webb *et al*., 1990; Schriefer *et al*., 1994b; Higgins *et al*., 1996).

Genome walking. One region of the *R. felis rompA* gene, the promoter and ATG start site, was amplified by the utilization

Table 1 Primers for amplification of *R. felis rompA* gene

Primer	Sequence	Reference
Promoter region and ATG start site		
GSP1 (RF321-292)	AGCTCCTCCCGTATCTACCACTGAACCTAA	This study
ASP1	GTAATACGACTCACTATAGGGC	Invitrogen
GSP1 (RF65-38r)	AGCTCCTCCCGTATCTACCACTGAACCTAA	This study
ASP2	ACTATAGGGCACGCGTGGT	Invitrogen
5′ region		
190·70	ATGGCGAATAATTCTCCAAAA	Regnery *et al.* (1991)
190·602n	AGTGCAGCAATTCGCTCCCCCT	
5′ repeat region overlap		
Rf 247f	AATAATTTTGCAGCAGGTCTTT	This study
Rf Repeat r	TGACTCAATGCTCCACTTTAGAT	
Repeat region		
675	CCAGACAGATGCTGCCATTAAGC	Walker *et al.* (1995)
2940	TTCCGATCTAGACTTCCTCCAAGC	
Repeat 3′ overlap		
Rf-Repeat f	AGGCGGTGATAATGTAGGTGTCT	This study
RfB5-27R	TTACTCGCAGCTCCAAAATCTAT	
3′ region (1·4 kb)		
RR-3622f	GCTGGAGGAAGCCTAGCTGCG	This study
RR-4999r	TGACCAACCGAATTAGCCGC	
3′ region (1·2 kb)		
F3-4936	GGTGGTCAGGCTCTGAAGCTAAAAC	Stenos & Walker (2000)
B21-6324	TGCAGTTTGATAACCGACAGTCTC	
3′ region (1·0 kb)		
6049	ACTGGTGGCACTATAGGTTTTGAC	P. A. Crocquet-Valdes, personal communication
7019	ATCGGCAGTTTTTCTAATAATAAT	

of a Genome Walker kit (Clontech) with a few modifications of the manufacturer's instructions. The primers are listed in Table 1. The DNA extract from the fleas was divided into five aliquots and subjected to digestion by five blunt-end-cutting restriction enzymes (*Dra*I, *Eco*RV, *Pvu*II, *Sca*I, *Str*I) at 37 °C overnight. The reaction mixtures were subjected to a series of phenol/chloroform extractions followed by chloroform extraction and ethanol precipitation. Adapters from the kit were ligated to the digested DNA samples at 16 °C overnight. The reaction was stopped by incubating the samples at 70 °C for 5 min, followed by the addition of Tris/EDTA buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8·0). Each sample was PCR-amplified in the primary reaction using the Advantage *Tth* polymerase kit (Clontech) with the primary *R. felis rompA* gene specific primer (GSP1) and adapter primer 1 (AP1) from the kit. The nested PCR was performed as above with the addition of 1 μl of the primary reactions as the template and the substitution of nested primers GSP2 and AP2 for the GSP1 and AP1 primers. The cycling parameters utilized were modified from those proposed in the manufacturer's instructions with the annealing temperature being 3 °C higher than that given in the instructions. The sequences of the primers are listed in Table 1.

Sequencing of the *rompA* gene. DNA extracts from the infected cat fleas were utilized for sequencing the *R. felis rompA* gene. Primers designed for amplification of the repeat region and 5′ region of the gene were from published sequences or were generated from sequence analysis of the *R. felis* PCR products (Table 1). The PCR amplification conditions for the reactions were 1 cycle at 95 °C for 5 min, 30 cycles of 1 min at 95 °C, 20 s at 48 °C, 3 min at 72 °C followed by 1 cycle of 5 min at 72 °C and 1 soak cycle at 4 °C. An aliquot of the PCR products was analysed by resolution on 1·3 % (w/v) agarose gels that were stained with ethidium bromide and visualized by a UV light source. The remaining PCR products were cloned using the TOPO TA Cloning kit (Invitrogen) and plated on selective media containing ampicillin and X-Gal/IPTG overnight at 37 °C. Positive clones were selected and grown in LB medium containing ampicillin. Plasmid DNA was isolated using the High Pure Plasmid Isolation kit (Roche Molecular Biochemicals) and digested with *Eco*RI according to the manufacturer's instructions. The restriction enzyme digests were analysed in a 1·3 % (w/v) agarose gel. Plasmids that contained DNA inserts were sequenced twice using an ABI automated sequencer with M13 and T7 sequencing primers (Gibco-BRL).

Genetic analysis. The primer design sequence alignment and preliminary comparison were facilitated through the use of the software programs GCG (Wisconsin Package, version 10.0, Genetics Computer Group, Madison, WI) and LASERGENE (DNASTAR, Madison, WI), which are built upon the CLUSTAL algorithm platform (Higgins & Sharp, 1989). The percentages of similarity were determined by the CLUSTAL method.

Phylogenetic analysis. Phylogenetic analyses were performed using the maximum-parsimony and distance program of the

PAUP 4.1 software (Swofford, 1998). Distance matrix analyses were generated with the Kimura two-parameter model for multiple substitutions (Kimura, 1980). Bootstrap values (Felsenstein, 1985) based on the analysis of 1000 replicates were determined to estimate the node reliability of the phylogenetic trees obtained by the parsimony, maximum-likelihood and distance methods.

The GenBank accession numbers of the 17 kDa protein gene sequences are: *Rickettsia australis*, M74042 (unpublished), M28480 (Anderson & Tzianabos, 1989); *Rickettsia massiliae* Mtu1^T, U11017 (unpublished); *Rickettsia parkeri*, U17008 (unpublished); *Rickettsia rickettsii*, M16486 (Anderson *et al.*, 1987); *R. typhi*, M28481 (Anderson & Tzianabos, 1989); and *R. felis*, AF195118 (this study). The GenBank accession numbers of all rickettsial *rompA* sequences compared are: *Rickettsia aeschlimannii* MC16^T, U83446, U43800 (Fournier *et al.*, 1998); *Rickettsia africae* ESF, U83436, U43790 (Fournier *et al.*, 1998); *Rickettsia akari* Kaplan, L01461 (Gilmore, 1993); *R. australis* PHS, AF149108 (Stenos & Walker, 2000); *Rickettsia conorii* Astrakhan, U83437, U43791 (Fournier *et al.*, 1998); *R. conorii* Israeli, U43797, U83441 (Fournier *et al.*, 1998); *R. conorii* Malish 7^T, U01028 (Crocquet-Valdes *et al.*, 1994); *R. conorii* Moroccan, U83443, U43798 (Fournier *et al.*, 1998); *Rickettsia honei* RB^T, AF018075, AF018076 (Stenos *et al.*, 1998); *Rickettsia japonica* U43795, U83442 (Fournier *et al.*, 1998); *R. massiliae* Bar29, U43792, U83444 (Fournier *et al.*, 1998); *R. massiliae* Mtu1^T, U83445, U43799 (Fournier *et al.*, 1998); *Rickettsia montanensis*, U43801, U83447 (Fournier *et al.*, 1998); *R. parkeri*, U43802, U83449 (Fournier *et al.*, 1998); *Rickettsia prowazekii*, M28482 (Anderson & Tzianabos, 1989); *Rickettsia rhipicephali*, U43803, U83450 (Fournier *et al.*, 1998); *R. rickettsii* M31227 (Anderson *et al.*, 1990); *Rickettsia sibirica* 246^T, U43807, U83455 (Fournier *et al.*, 1998); *R. sibirica* mongolotimonae, U43796, U83439 (Fournier *et al.*, 1998); *Rickettsia slovaca* 13-B, U43808, U83454 (Fournier *et al.*, 1998); and *R. felis*, AF191026 (this study).

Transmission electron microscopy. Adult fleas and larvae were anaesthetized on dry ice, their heads were removed and they were immediately dissected in a drop of fixative. Midguts with adjacent tissues were fixed in a mixture of 1·25% (v/v) formaldehyde, 2·5% (v/v) glutaraldehyde, 0·03% trinitrophenol and 0·03% $CaCl_2$ in 0·05 M cacodylate buffer, pH 7·3 (Ito & Rikihisa, 1981), post-fixed in 1% OsO_4 in the same buffer, stained *en bloc* with 1% uranyl acetate in 0·1 M maleate buffer (0·1 M maleic acid), pH 5·2, dehydrated in ethanol and embedded in Spurr low-viscosity epoxy resin (Polysciences). Ultrathin sections were cut on a Reichert Ultracut S ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips 201 electron microscope at 60 kV.

RESULTS

Genetic analysis

To verify that the DNA isolated from the fleas contained *R. felis* genomic DNA, RFLP analysis was performed on the 17 kDa protein gene PCR product. The flea DNA extract amplified using rickettsial specific primers yielded a 434 bp product. This product was then amplified by nested primers resulting in a 231 bp product. The nested product was then digested using restriction enzymes (*Alu*I and *Xba*I) and was resolved by agarose gel electrophoresis (data not shown). This method yielded a pattern that was characteristic of *R. felis* and was distinct from that of the *R. typhi* 17 kDa protein gene PCR product (Schriefer *et al.*, 1994a; Higgins *et al.*, 1996). Once the DNA was confirmed to be that of *R. felis*, phylogenetic analysis using parsimony was conducted on the sequence (Table 2). Due to the limited number of 17 kDa protein genes in GenBank, only nine *Rickettsia* species were analysed. Kimura two-parameter model analysis placed *R. felis* in the SFG of rickettsiae with only 5·3% divergence from *R. australis*. The *R. felis* 17 kDa protein gene was only 5·3–6·6% divergent from the other SFG rickettsiae, but was 11·3 and 11·5% divergent from *R. typhi* and *R. prowazekii*, respectively.

Initially, the sequence was generated for the *R. felis rompA* gene through the utilization of primers designed for the sequencing of the *R. australis rompA* gene (Bouyer *et al.*, 1999; Stenos & Walker, 2000). This generated a 1279 bp fragment. The amplification strategy for the rest of the *R. felis rompA* gene involved the use of published primers that were shown to have been effective for delineating other rickettsial *rompA* genes (Regnery *et al.*, 1991; Walker *et al.*, 1995). A set of primers was also developed by this laboratory from the published *R. rickettsii rompA* sequence (Anderson *et al.*, 1990) and from the generated *R. felis* sequence. A region of the *R. felis rompA* gene sequence that was difficult to amplify was obtained by genome walking. This sequencing strategy yielded a DNA sequence of 5513 bp with a G+C content of 39·49 mol% (data not shown). The gene had an ORF of 1860 bp (G+C content of 40·27 mol%) that was found to contain four repeat units, which consisted of two complete repeat units (225 and 216 bp) and two repeat units containing deletions of 6 and 69 bp, resulting in altered units of 219 and 147 bp in size, respectively. The promoter region of the *R. felis rompA* gene was found to be similar to that of the *R. australis rompA* gene (Stenos & Walker, 2000). The coding sequences for the putative ribosome-binding site, −10 sequence and −35 region shared 100% homology between those two species. Genetic analysis of the full length *R. felis rompA* gene ORF (1860 bp) and its comparison with other SFG *rompA* gene ORFs that have been sequenced was problematic due to the fact that the *R. felis* gene contained a premature stop codon, thereby resulting in a much smaller coding region than *R. australis* (6320 bp), *R. conorii* strain Malish 7^T (6065 bp) and *R. rickettsii* (6749 bp). The *R. felis rompA* gene ORF was found to have 51·6, 48·6 and 37·9% similarity to *R. rickettsii*, *R. conorii* and *R. australis*, respectively (data not shown). The marked divergence from *R. australis* is explained by its having the most divergent of rickettsial *rompA* repeat domains (Stenos & Walker, 2000). It was determined that the most effective means to analyse the *R. felis rompA* gene would be to convert the gene into protein domains and combine the areas of interest outside of the repeats. The rickettsial rOmpA proteins were divided using the pattern

Table 2 Kimura two-parameter model of divergence of 17 kDa protein gene of *Rickettsia* species

Species	*R. felis*	*R. typhi*	*R. conorii*	*R. montanensis*	*R. parkeri*	*R. prowazekii*	*R. rhipicephali*	*R. rickettsii*
R. felis								
R. typhi	0·113262							
R. conorii	0·058047	0·109749						
R. montanensis	0·064669	0·113315	0·018914					
R. parkeri	0·066177	0·121191	0·008193	0·014556				
R. prowazekii	0·115637	0·05131	0·114369	0·11255	0·122627			
R. rhipicephali	0·06216	0·115125	0·02068	0·012448	0·016631	0·12291		
R. rickettsii	0·061808	0·113979	0·00777	0·022668	0·011931	0·118665	0·024479	
R. australis	0·053777	0·114894	0·039453	0·039664	0·041584	0·122358	0·037438	0·043379

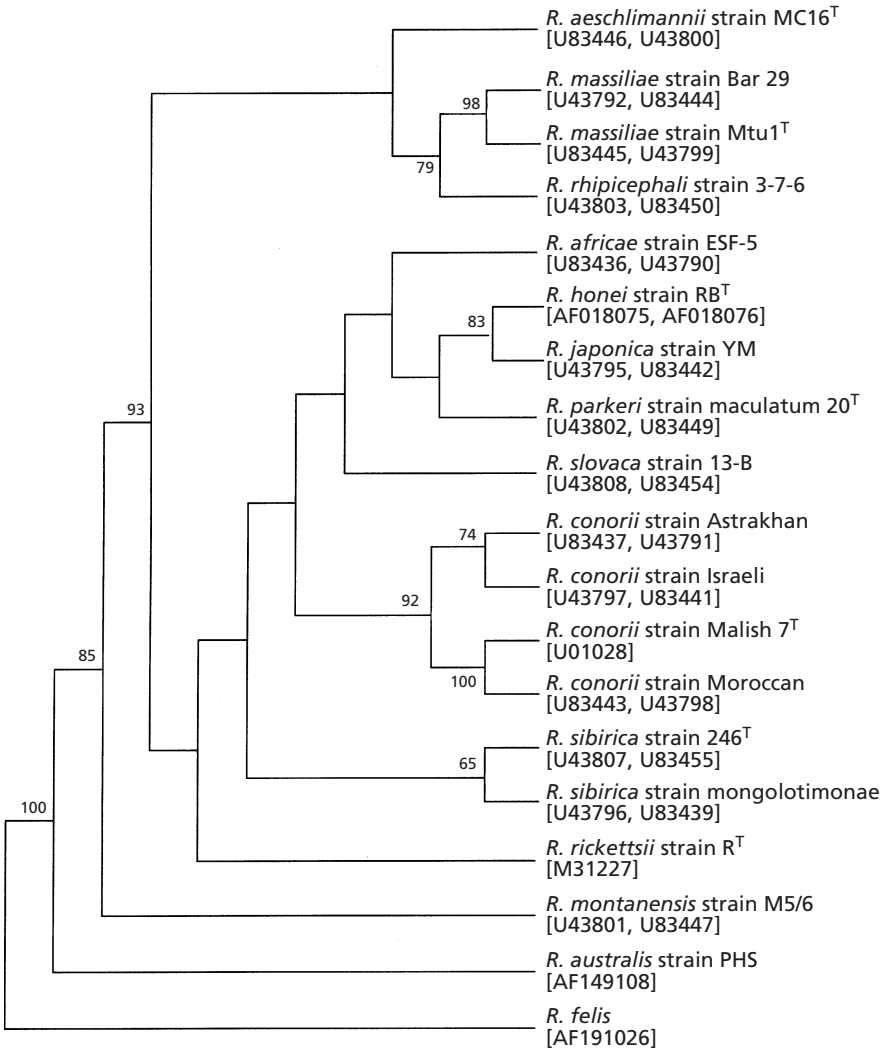

Fig. 1. Combined phylogenetic analysis of domains I and III of rOmpA proteins. A 340 aa polypeptide for each of the listed *Rickettsia* species was created by combining domain I (197 aa) with domain III (140 aa) of their rOmpA proteins. The length of all the proteins was designed to match the length of the *R. felis* protein (321 aa). The unrooted tree shown was generated from parsimony analysis of the data set. The node values were obtained from 1000 bootstrap replications to ensure certainty.

suggested by Anderson *et al.* (1990). The domains were linked using the method of Fournier (1998). Domain I of each rOmpA protein started at the initial methionine (residue 1) of the ORF and ended at the beginning of the repeat region. The domain I–domain III rOmpA fusions were 340 aa residues in length, which corresponds with the size of the protein that would be encoded by *R. felis* minus the repeat domain. This approach allowed the protein segments analysed to be closer in size: *R. felis* domain I being 206 aa residues in length; *R. rickettsii* and *R. conorii*, 211 aa residues; and *R. australis*, 265 aa residues. The entire repeat region of each of the rOmpA proteins was considered as domain II. The hydrophobic region that immedi-

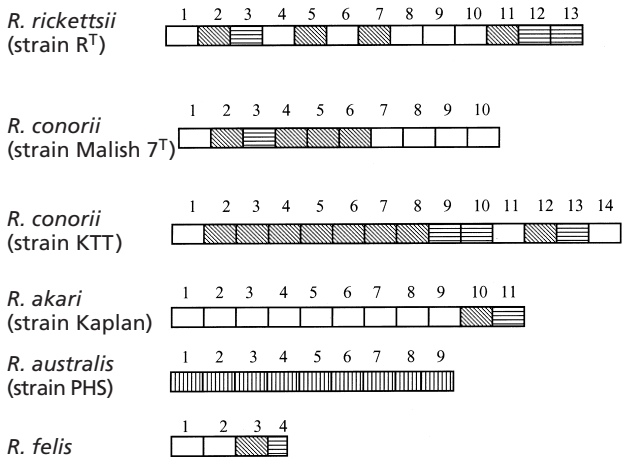

Fig. 2. Comparison of arrangement and length of sequenced *Rickettsia* species rOmpA repeat regions (domain II). White squares represent type I repeats (75 aa), slanted lines represent type IIa repeats (72 aa), horizontal lines represent type IIb repeats (72 aa) and vertical lines represent type III repeats (85 aa), which thus far have been observed only in *R. australis*. Domain II of *R. felis* is smaller in length than other *Rickettsia* species domain II.

ately follows the repeat domain is domain III. This region contained the premature stop codon in the *R. felis rompA*. Domain IV of the *R. felis* rOmpA, which was not used in our analysis because it contained several stop codons, consisted of primarily hydrophilic regions.

Amino acid sequences of *R. felis* rOmpA were analysed for percentage similarity utilizing the CLUSTAL algorithm (Higgins & Sharp, 1989). Comparison of the *R. felis* rOmpA domain I with that of other *Rickettsia* species showed 36·4 % similarity with *R. australis*, 40·8 % similarity with *R. conorii* (strain Malish 7^T) and 41·7 % similarity with *R. rickettsii* at the amino acid level. Phylogenetic trees constructed from parsimony and distance analysis both indicated that *R. felis* was nearer to *R. australis* (Fig. 1). This was confirmed by the Kimura two-parameter model (Kimura, 1980; data not shown).

The repeat region (domain II) of the *R. felis* rOmpA is unique by several criteria when compared to other rOmpA repeat domains (Fig. 2). The repeat region is smaller than in other published naturally occurring species as it contains only three complete or near-complete repeat units and one partial repeat unit. The first repeat is a type I. The second repeat unit is homologous to a type I repeat with the deletion of 2 aa being the only difference. The third repeat is of type IIa and is 72 aa. Comparison of the partial fourth repeat to a type IIb unit showed that it had a deletion of 23 aa. Domain III of the *R. felis* rOmpA protein is most similar to *R. australis* (46·2 %; data not shown).

Recently in a study conducted by this laboratory (Moron *et al.*, 2001), phylogenetic analysis (parsimony, distance and maximum-likelihood) of the DNA sequences of rickettsial *rompB* genes was performed. The *R. felis rompB* sequence was found to be less divergent from the SFG rickettsiae (10–13 % divergence) than from the typhus group (TG) rickettsiae (18 %). Analysis of the *R. felis* rOmpB protein by the CLUSTAL algorithm confirmed that *R. felis* is most similar to the SFG rickettsiae.

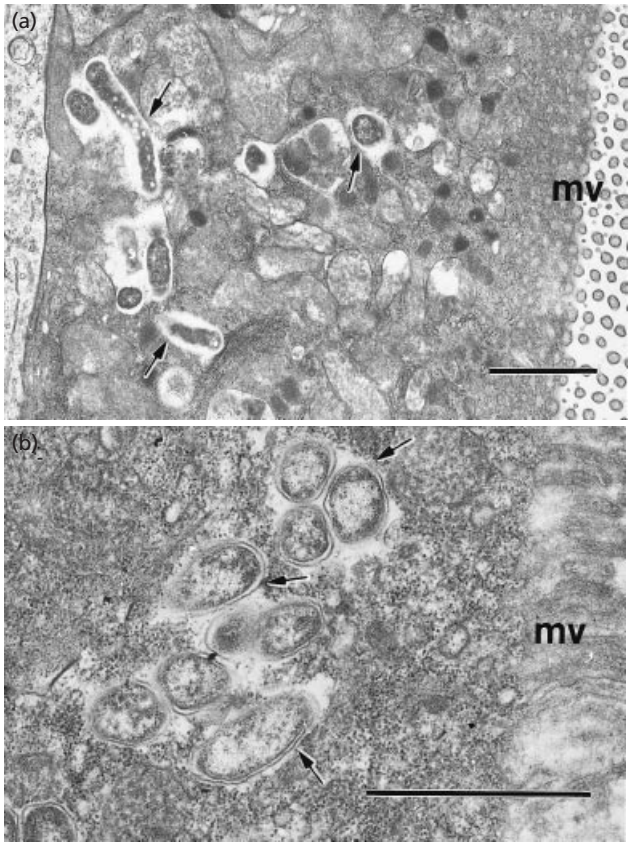

Fig. 3. *Rickettsia felis* in midgut epithelial cells of the cat flea. Bars, 1 μm. (a) In midgut epithelial cells of larvae, rickettsiae with dense cytoplasm (arrows) are located free in the cytosol surrounded by a clear space. Some rickettsiae have intracytoplasmic vacuoles. mv, Epithelial cell microvilli. (b) In a midgut epithelial cell of an adult flea, rickettsiae surrounded by two trilaminar membranes (arrows) are typically localized free in the cytosol. mv, Microvilli.

Electron microscopy

In adult cat fleas, rickettsiae were found in midgut epithelial cells and in underlying tissues, including muscles, and in oocytes. In larvae, rickettsiae were mostly localized in midgut epithelial cells. Rickettsiae were typically located free in the cytosol surrounded by electron-lucent clear spaces (Fig. 3). They varied in length, width and density of the cytoplasm, some rickettsiae in larvae having dense cytoplasm (Fig. 3a). Also, rickettsiae in larvae were observed to have intracytoplasmic vacuoles (Fig. 3a).

DISCUSSION

Traditionally, organisms of *Rickettsia* species are propagated and analysed by microimmunofluorescence serotyping, mAb serotyping and comparison of proteins by SDS-PAGE and Western immunoblot comparison (Beati *et al*., 1992, 1997). Although effective, the serotyping methods are cumbersome and based on limited epitopes that are determined by small portions of the genome. Problems can also arise with the SDS-PAGE and Western immunoblot methods because the protein profiles of particular strains of a species can differ greatly (Walker *et al*., 1992). Rickettsial species, such as *Rickettsia peacockii* and *Ehrlichia ewingii*, that have thus far proven to be resistant to cultivation, have been classified by molecular analysis of selected genes (Anderson *et al*., 1987; Neibylski *et al*., 1997). Since the reliable continuous cultivation of *R. felis* has yet to be reported, it is felt that genetic analysis would be the best method for its classification, as was done for *R. peacockii* and *E. ewingii*.

R. felis has traditionally been placed within the TG of *Rickettsia* on the basis of reactivity of *R. felis* antigens with antibodies to *R. typhi* and the previous association of the TG with insects and the SFG with acarines (Adams *et al*., 1990; Azad *et al*., 1992). In contrast, data from genetic studies would place *R. felis* in the SFG (Azad *et al*., 1992; Roux & Raoult, 1995; Stothard & Fuerst, 1995). For example, comparison of a 381 bp fragment of the CS gene of *R. felis* and several other rickettsial species indicated that there were 24 bp differences between *R. felis* and *R. prowazekii*. This was similar to the 25 bp difference between *R. rickettsii* and *R. prowazekii* (Higgins *et al*., 1996). Higgins and others also reported that there was a 32 bp difference between *R. felis* and *R. typhi*. There was only an 11 bp difference between *R. prowazekii* and *R. typhi*, both members of the TG, indicating that *R. felis* is genetically a different species. The relatively small difference, only 13 bp, between *R. felis* and *R. rickettsii* further supports the placement of *R. felis* in the SFG. The use of the CS gene has been recognized as a useful tool for analysis of the genus *Rickettsia* (Roux *et al*., 1997). Phylogenetic analysis of the 16S rRNA gene of 14 rickettsial species indicated that *R. felis* forms a clade with *R. akari* and *R. australis* (Stothard & Fuerst, 1995). *R. felis* is divergent from the aforementioned bacteria by only 1·2 and 0·4 %, respectively. Our analysis of the *R. felis* 17 kDa protein gene also indicates that *R. felis* does not belong in the TG of rickettsiae. *R. felis* was found to have an 11 % divergence from the TG, which corresponds with the 10–12 % divergence between the SFG and TG. These results were confirmed by a comprehensive study involving molecular analysis of multiple genes of several *Rickettsia* species, including *R. felis* (Andersson & Andersson, 1999). Phylogenetic analysis of the predicted amino acid sequences of the *ftsY*, *polA* and *dnaE* gene products also showed that *R. felis* is a member of the SFG. It was interesting to note that the genes used for the construction of the aforementioned tree were neighbouring genes representing a segment of slightly more than 9000 bp of the *R. felis* genome.

Recently, a dengue-like illness was described that was caused by an unknown SFG rickettsia (Zavala-Velazquez *et al*., 1996). Since it was suspected that the aetiological agent in those cases was perhaps *R. felis*, it was decided to investigate whether the bacterium harbours a gene that encodes rOmpA (Bouyer *et al*., 1999). This was significant because only members of the SFG have been found to contain *rompA*. The complete sequence was obtained and the *R. felis rompA* gene was characterized, thus providing evidence that *R. felis* is a *Rickettsia* of the SFG. A DNA sequence of *R. felis* 5513 nt in length contained sequences homologous to *rompA* including an ORF of 1860 bp which would encode a protein of a predicted size of 60·5 kDa. Although the premature stop codon resulted in an estimated size of the *R. felis* rOmpA much smaller than the observed and predicted sizes of the other known rickettsial rOmpA proteins, it is not a truly unexpected phenomenon. It has been reported that two SFG *Rickettsia* species, *R. rhipicephali* and *R. sibirica*, contained termination codons in the middle region of the *metK* gene (Andersson & Andersson, 1999). *R. felis* was also found to have a deletion of 3018 bp within the *metK*/*dnaE* intergenic region, which is substantially greater than that seen with other *Rickettsia* species and 50 bp in the *fmt*/*rrl* intergenic region (Andersson & Andersson, 1999). This phenomenon of deletion of a gene segment was also observed in the *R. felis rompA* repeat region. This region consisted of three complete or near-complete repeat units and one partial repeat, which makes the region smaller than that of the other SFG rickettsiae for which the sequencing of domain II has been reported, namely *R. rickettsii* (13 repeats) and *R. conorii* strain Malish 7^T (10 repeats) and Kenya tick typhus strain (14 repeats), *R. akari* (11 repeats) and *R. australis* (9 repeats) (Fig. 2). Further analysis of the *R. felis* repeat units showed that two of the repeats (the second and fourth) contained a different number of amino acids than previously observed in a rickettsia (Anderson *et al*., 1990; Gilmore & Hackstadt, 1991; Gilmore, 1993; Crocquet-Valdes *et al*., 1994). Type I repeats consisted of 75 aa; whereas, type II repeats contained 72 aa. The newly designated type III repeats, which are unique to *R. australis*, contained 85 aa (Stenos & Walker, 2000). *R. felis* contained a repeat unit of 73 aa that, if not for a deletion of 2 aa, would have been a typical complete type I repeat. There has also been a report of the deletion of most of the repeat domain of the *rompA* gene of a laboratory-passaged strain of *R. rickettsii* (Matsumoto *et al*., 1996). This strain was found to have only one repeat unit within the mutated repeat domain instead of the expected 13 repeat units. Any influence that the aforementioned variations may have exerted upon serological assays is unknown at this time but is currently being studied by our group.

The evidence that *R. felis* contained a gene that encodes

rOmpA suggests that *R. felis* is a novel *Rickettsia* species in the SFG. As stated previously, rOmpA distinguishes members of the SFG from TG rickettsiae, and the very presence of the gene in *R. felis* indicates that its current classification should be revised. In addition our phylogenetic analysis of the merged rOmpA proteins from 19 species of *Rickettsia* places *R. felis* near *R. australis*.

Although the existence of the *R. felis* rOmpA is of importance, we are left to ponder the reactivity of *R. felis* with antibodies stimulated by *R. typhi* (Azad *et al*., 1992). It could be debated that the first identification of *R. felis* in the cat flea was in fact *R. typhi*, but this seems unlikely as shown by the distinct RFLP pattern of the *R. felis* CS and 17 kDa protein genes and their DNA sequences (Higgins *et al*., 1996; Azad *et al*., 1992). Perhaps a more plausible theory is that *R. felis* is closer to the common *Rickettsia* ancestor on the evolutionary scale than any of the previously analysed species. This theory could be supported by *R. felis* containing features of both the SFG and TG and also the observations that several genes of *R. felis*, including *rompA*, have been found to contain large deletions of nucleotides. This is one of the first reports of a potentially dying virulence gene in the *Rickettsia* species.

The expression of the *R. felis rompA* gene product is currently being investigated by our laboratory. Ultimately, the analysis of *R. felis* will allow the solution of several evolutionary questions. It is proposed that this bacterium, formerly known as the ELB agent, be designated *Rickettsia felis* sp. nov., type strain *Ctenocephalides felis*-LSU^T, and be placed in the SFG of rickettsiae. The type strain is maintained in an infected flea colony by the Department of Entomology, Louisiana State University Agricultural Center.

Description of *Rickettsia felis* sp. nov.

Rickettsia felis [felis. L. gen. n. *felis* of the cat) is named to recognize the discovery and origin of the bacterium in the cat flea (*Ctenocephalides felis*), a parasite of the cat (*Felis domesticus*) and opossum (*Didelphis marsupialis*) (Adams *et al*., 1990; Schriefer *et al*., 1994; Higgins *et al*., 1996; Matthewman *et al*., 1997].

The bacterium was first observed by electron microscopy of tissues of the cat flea and was reported to have the typical rickettsial membrane ultrastructure (Adams *et al*., 1990). The organism is 0·25–0·45 μm in diameter by 1·5 μm in length and has been observed by this laboratory in both adult fleas and their larvae (Fig. 3a, b). The organism is maintained in the flea by vertical (transovarial) transmission (Azad *et al*., 1992). At the time of writing, reliable propagation of *R. felis* in culture has not been reported.

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