PHYLOGENETIC ANALYSIS OF THE *ROMPB* GENES OF *RICKETTSIA FELIS* AND *RICKETTSIA PROWAZEKII* EUROPEAN-HUMAN AND NORTH AMERICAN FLYING-SQUIRREL STRAINS

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Abstract. The rickettsial outer membrane protein B (*rompB*) gene encodes the major surface antigens of *Rickettsia* species. We undertook sequencing and molecular analysis of the *rompB* gene of *Rickettsia felis* and a comparison with its homologs in spotted fever group (SFG) and typhus group (TG) rickettsiae, including the complete sequences of two North American flying squirrel strains and two European human strains of *Rickettsia prowazekii*. We sequenced 5,226 base pairs (bp) of the *R. felis rompB,* encoding a protein of 1,654 amino acids. We also sequenced 5,015 bp of *rompB* of the flying squirrel strains, encoding a protein of 1,643 amino acids. Analysis of the *R. felis rompB* gene sequence showed 10–13% divergence from SFG rickettsiae and 18% divergence from the TG rickettsiae. The *rompB* of all sequenced strains of *R. prowazekii* showed an overall similarity of 99.7–99.9%.

INTRODUCTION

One of the rickettsial major surface proteins has been referred to as rickettsial outer membrane protein B (rOmpB). The interest in this protein results from its major quantity and outer membrane location and the strong immune response observed after infection, suggesting an important yet undefined function under evolutionary selective pressure.[1,2] Although the rOmpB encoding gene (*rompB*) is present in both spotted fever group (SFG) and typhus group (TG) rickettsiae, it has been neglected as a phylogenetic tool. Genotypic relationships among *Rickettsia* species have been analyzed previously based on the alignment of two housekeeping genes, the 16S rRNA gene and citrate synthase (*gltA*) gene, and the rickettsial outer membrane protein A (rOmpA) encoding gene.[2–4]

Louse-borne typhus is a reemerging cause of morbidity and mortality among isolated impoverished populations in tropical locations. *Rickettsia prowazekii* is the etiologic agent of epidemic typhus that clinically has involved only humans and their body lice. However, in 1963 an extra-human reservoir of the agent of epidemic typhus was discovered based on serologic data and subsequently isolates of *R. prowazekii* from flying squirrels in the eastern United States.[5] Its transmission among flying squirrels by their own lice and fleas has also been described.[5,6] Since then, reports of human cases of epidemic typhus in the eastern United States have appeared in which flying squirrels were likely to have been the source.[7–9] A preliminary report from this laboratory described a phylogenetic analysis of the *rompB* gene of two *R. prowazekii* North American flying squirrel strains from Florida and Virginia and compared them with its homolog in two human strains of *R. prowazekii,* Madrid E and Breinl, which were isolated from humans in Poland just after World War I and in Spain during World War II, respectively.[10]

Recently, the laboratory of Azad characterized another insect-transmitted rickettsia responsible for a zoonosis in North America. A flea-borne rickettsia, previously referred to as the ELB agent, has been implicated as a cause of human disease.[11–16] Higgins proposed that the organism be designated as *Rickettsia felis*.[11] The use of the appellation *felis* recognizes the origin of this bacterium in the cat flea, *Cteno-cephalides felis*.[12] *Rickettsia felis* has been considered a member of the TG rickettsiae based on its reactivity with anti-*Rickettsia typhi* antibodies. However, genetic analysis of the 16S rRNA, citrate synthase, and *rompA* genes placed *R. felis* as a member of the SFG.[3,12,13] We consider the *rompB* gene a possible useful tool for phylogenetic analysis to support this classification.

In this study, we undertook the sequencing of the *rompB* gene of *R. felis* and of two North American flying squirrel strains of *R. prowazekii,* insect-transmitted pathogens causing zoonoses in North America, because we considered that this gene, under the selective pressure of the immune response, offers the best opportunity to observe genetic divergence. We inferred the phylogenetic relationships among these rickettsiae and other species with the sequences of the *rompB* gene previously reported, including two human strains of *R. prowazekii* from Europe.

MATERIALS AND METHODS

***Rickettsia prowazekii* strains, cultivation, and nucleic acid preparation.** *Rickettsia prowazekii* (Gv F-12, Gv V-257, and Breinl strains) were generously provided by G. A. Dasch (Naval Medical Research Center, Bethesda, MD). Strains Gv F-12 and Gv V-257 were isolated from *Glaucomys volans* flying squirrels in Florida and Virginia, respectively.[5] They were propagated in Vero cell monolayers in Eagle's minimum essential medium supplemented with 4% fetal calf serum and 2 mM glutamine at 34°C. The cells were harvested when the degree of infection as estimated by Gimenez staining[16] was high (70% or greater). Rickettsial cultures were centrifuged (22,000 × *g* for 20 min), resuspended in sucrose-phosphate-glutamate (SPG) buffer (0.21 M sucrose, 0.038 M potassium phosphate monobasic, 0.072 M potassium phosphate dibasic, and 0.049 M glutamate, pH 7.0) and stored at −80°C until required. Cells were lysed by overnight incubation in a mixture of 0.5% sodium dodecyl sulfate and proteinase K (100 μg/mL) in an Eppendorf tube at 37°C. A phenol:chloroform:isoamyl alcohol (25:24:1, v/v) extraction was carried out, followed by DNA precipitation in 2 volumes of ethanol and 0.5 volumes of 7.5 M ammo-

nium acetate and pelleting by centrifugation (22,000 × *g* for 2 min). The pellet was rinsed with 70% ethanol, air dried, and resuspended in Tris hydrochloride-EDTA buffer (TE), pH 8.0.

Source of *R. felis*-infected fleas and DNA extraction. The infected cat fleas were maintained in the persistently-infected laboratory colony of the Department of Entomology, Louisiana State University Agricultural Center.[14] The prevalence of infection in this flea colony has been determined to be 86% by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis of the *Rickettsia* 17 kDa antigen gene.[15] Fleas were pooled in groups of 10–12. Each pool of fleas was placed in 70% ethanol for 15 min followed by washing in phosphate buffered saline (PBS 8.1 mM sodium phosphate dibasic, 1.47 mM potassium phosphate monobasic, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.1). After the PBS wash, each flea pool was placed into a sterile microcentrifuge tube with 200 μL of PBS and pulverized with a sterile micropestle (Brinkmann, Westbury, NY). Proteinase K (100 μg/mL) and sodium dodecylsulfate (SDS) (0.5% w/v) were added to the suspension, which was placed in a 37°C water bath for one hr followed by a 10 min incubation at 56°C. A series of phenol/chloroform extractions were performed on the homogenate followed by a single chloroform extraction. The DNA was precipitated in two volumes of cold absolute ethanol with 0.1 volume of 2 M NaCl and pelleted. The pellet was rinsed with 70% ethanol, air dried, and resuspended in TE buffer, pH 8.0.

PCR amplification. PCR amplification of the *rompB* gene of the Florida and Virginia strains of *R. prowazekii* was performed using the oligonucleotide pairs: Rp 25f (AGTGTGGGA TATCTTGACCTCGTA, positions 25–48) and Rp 1217r (ACCGCCTAAACCAACA TCAACTAT, positions 1217–1194); Rp 1099f (TGTAGGATTTATTGT-TAGTGTTGA, positions 1099–1122) and Rp 3162r (GCGTCAAAATCTGTCCCTGCTA, positions 3162–3141); Rp 2902f (ATCGCTTGGAAGTGATAACAGTA, positions 2902–2924) and Rp 5003r (CTTTTAAAGTACCTT-GATGTGC, positions 5003–4982); and Rp 4625f (CCA-ATGGCAGGACTTAG, positions 4625–4641) and Rp 5047r (TATAAACTTACT AAAATCACAAAT, positions 5047–5024). They were developed based on the *R. prowazekii* (Breinl strain) *rompB* sequence reported in GenBank (accession number M37647). The PCR cycling conditions were performed in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) and consisted of 30 cycles each of 1 min at 95°C, 1 min at 48°C, and 2 min at 72°C, and a final extension cycle of 5 min at 72°C. PCR products were analyzed by electrophoresis in a 1% agarose gel and by visualization using staining with ethidium bromide.

Polymerase chain reaction amplification of the *R. felis rompB* gene was performed using oligonucleotide primers designed based on *Rickettsia japonica* (GenBank accession number AB003681) and *R. prowazekii* (Breinl strain) (GenBank accession number M37647) sequences: Rj 1f (TGATAAGGGATAACGCCAATGTG, positions 1–23) and Rj 1039r (ACTAA TCTACCCGTACCGTCTGTT, positions 1062–1039); Rj 952f (CGGTCAAGGTTT CATGTTCAA-TAC, positions 952–975) and Rj 2759r (ACCACTACGT-TACCGGGA CCAGA, positions 2781–2759); Rj 2759f

(TCTGGTCCCGGTAACGTAGTGGT, positions 2759–2902) and Rp 2902r (ATACTGTTATCACTTCCAAGCGAT, positions 2925–2902); and Rp 2902f (ATCGCTTGGAAGT-GATAACAGTAT, positions 2902–2925) and Rp 5003r (CTTTTAAAGTACCTTGATGTGC, positions 5024–5003). We also used a *R. felis*-specific primer, Rfe 5049 (GTCTTA-TAATATAGGCTTAAGTGC, positions 5049–5072) and RFeIRr (GGGCTTTTTTCTTGATCAAGTTATAAA), a primer designed based on the conserved intergenic region of *rompB-ccmf* of *R. japonica, R. typhi,* and *R. prowazekii*. The PCR cycling conditions were performed in a DNA Thermal Cycler and consisted of 30 cycles each of 1 min at 95°C, 1 min at 48°C (or 55°C depending on the primer), and 2 min at 72°C, followed by a final extension cycle of 5 min at 72°C.

Cloning and sequencing. All PCR products were cloned using the TOPO™ TA Cloning Kit (Invitrogen, Carlsbad, CA), and the transformants were plated on selective media containing ampicillin and X-gal overnight at 37°C. Positive clones were selected and grown in Luria-Bertani (LB) medium containing ampicillin. Verification that the clones contained inserts was accomplished by digestion of plasmid DNA with *Eco RI* and separation in 1.3% agarose gels. Plasmid DNA was isolated using the Rapid Prep Kit (Boehringer Manheim, Indianapolis, IN). Plasmids containing DNA inserts were sequenced for both strands using Perkin-Elmer Ready Reactions and Amplitaq FS with a Perkin-Elmer ABI Prism 377 Automated DNA Sequencer (Foster City, CA).

Data analysis. The *rompB* sequences were translated into amino acid sequences using Lasergene software package (DNAstar, Madison, WI). The *rompB* sequences and rOmpB amino acid sequences were aligned using the Megalign program of Lasergene software package. DNA sequences were compared using the CLUSTAL method[17] for pairwise alignment with a gap penalty of 5. A gap penalty of 3 was used for amino acid comparison. Phylogenetic analyses were performed using PAUP 4.0 software.[18] Distance matrix analysis was generated by the Kimura 2 parameter for multiple substitutions. Bootstrap values based on analysis of 1,000 replicates were performed to estimate the node reliability of the phylogenetic trees obtained by the parsimony, maximum-likelihood, and distance methods.[19]

GenBank accession numbers for the genes characterized in this study are *R. felis rompB* (AF182279), *R. prowazekii* Gv F-12 strain (AF211820), *R. prowazekii* Virginia Gv V-257 strain (AF211821), and *R. prowazekii* Breinl strain (AF161079). The other *Rickettsia* species utilized for genetic analysis are listed in Table 1.

No human subjects or experimental animals were used in this research.

<div align="center">RESULTS</div>

PCR amplification and sequencing. Utilization of the primers previously mentioned generated a 5,226 bp PCR product with an open reading frame (ORF) of 4,965 bp, encoding a protein with the predicted size of 167 kDa for the *R. felis* rOmpB.

The *rompB* sequences of the *R. prowazekii* strains isolated from flying squirrels from Florida and Virginia were determined. We sequenced a 5,015 bp product which included an ORF of 4,932 bp, encoding a protein of 1,643 amino acids.

Species	GenBank accession number	Length (nt)	Length (AA)
Spotted fever group rickettsiae			
Rickettsia australis	AF149109	3909	1304
Rickettsia conorii	AF149110	3906	1303
Rickettsia japonica	AB003681	5209	1735
Rickettsia rickettsii	X16353	5164	1720
Typhus group rickettsiae			
Rickettsia typhi	L04661	5081	1692
Rickettsia prowazekii Madrid E strain	AJ235273	4932	1643

nt = nucleotides; AA = amino acids.

During these sequence analyses and comparison with its homolog in *R. prowazekii* Madrid E and Breinl strains, an error for the *R. prowazekii* (Breinl strain) *rompB* remitted in GenBank (accession number M37647) was suspected.[20] Thus, we amplified and sequenced the Breinl strain *rompB*, a 5,015 bp gene with an ORF of 4,932 bp, and remitted to GenBank the corrected sequence (accession number AF161079). Our sequencing data revealed that there were nucleotide errors in the previous sequence and showed the presence of an additional adenine at position 138 of that sequence, which moved the ATG translation start 96 bp upstream from the original sequence (data not shown). The presence of this additional adenine was reported previously by Gilmore for *R. prowazekii*.[1]

Sequence comparison. Variations in the size of the *rompB* sequences were observed among the rickettsiae analyzed. As previously mentioned, these sequences were compared using the CLUSTAL method for pairwise alignment. Table 2 shows the levels of DNA sequence divergence of *rompB* among the *Rickettsia* species. Analysis of the *R. felis rompB* DNA sequence showed it was similar to the other SFG rickettsiae, displaying a 10–13% divergence, compared to an 18% divergence from TG rickettsiae. The *rompB* of all the sequenced strains of *R. prowazekii* differed only slightly from one another, between 0.1 and 0.3%. All nucleotide differences among the *R. prowazekii* isolates were due to base substitutions, as shown in Table 3.

Table 4 shows the levels of amino acid sequence similarity and divergence among the *Rickettsia* species. *Rickettsia felis*

showed 15–18% divergence from the other SFG rickettsiae compared to 28% divergence from the TG rickettsiae. *Rickettsia prowazekii* isolates differed 0.1–0.6% from one another. Of the 1,643 amino acids of rOmpB, we found only 2–11 amino acid differences between strains (Table 5).

Phylogenetic analysis. The phylogenetic trees obtained with the three tree-building analytic methods, as described in Materials and Methods, showed similar organization for all rickettsiae. All the nodes were well supported with bootstrap values > 70% (Figure 1). *Rickettsia felis* appears to be more closely related to the SFG taxa than to the TG taxa, a characteristic that it shares with *Rickettsia australis.* All the *R. prowazekii* strains showed a very close evolutionary relationship.

DISCUSSION

Rickettsia prowazekii is the etiologic agent of a reemerging infectious disease in South America, Africa, and Russia.[21–24] Louse-borne typhus has been a persistent problem in Peru for centuries. Low socioeconomic conditions and certain cultural patterns are closely associated with the periodic reemergence of this potentially fatal infectious disease.[24] In Africa, outbreaks have historically occurred in Burundi, Rwanda, southwest Uganda, and Ethiopia.[21] In 1997, a huge outbreak of epidemic typhus occurred in Burundi among refugees displaced following the onset of civil war.[21] In the same year a small outbreak occurred in Russia, where political transformation has been accompanied by profound changes in social conditions.[23]

Until now the origin of typhus has been unclear. Some historians such as Haeser and Hecker contended that typhus was first described by Thucydides during the Plague of Athens (430–425 BC).[25] Zinsser noted that Hippocrates' *First Book of the Epidemion* contained a description of a patient who might have had typhus. Older chronicles which predated the fourteenth century did not accurately describe the signs and symptoms of disease, and most epidemic diseases were referred to as "plague," which could be presumed to be typhus, bubonic plague, dysentery, yellow fever, cholera, scurvy, meningococcal disease, or smallpox.[25] The general consensus among historians is that typhus was carried from the East or from Africa to Europe during the first century and that it eventually reached the Spanish peninsula by the

TABLE 2

DNA sequence pair distances of the *rompB* of rickettsial species. Percent similarity is shown in the upper triangle and percent divergence in the lower triangle

	Rf	Rj	Rr	Ra	Rc	Rt	RpM	RpB	RpF	RpV
Rf		89.3	88.7	86.7	89.3	81.4	81.9	81.8	81.8	81.7
Rj	10.7		95.7	86.7	96.5	79.4	79.2	79.2	79.2	79.0
Rr	11.3	4.3		86.3	97.9	79.2	78.7	78.6	78.6	78.5
Ra	13.4	13.3	13.7		86.9	78.1	77.9	77.7	77.9	77.3
Rc	10.7	3.5	2.1	13.1		79.6	79.6	79.5	79.7	79.5
Rt	18.6	20.6	20.8	21.9	20.4		92.0	92.0	91.9	91.9
RpM	18.1	20.8	21.3	22.1	20.4	8.0		99.9	99.9	99.8
RpB	18.2	20.8	21.4	22.3	20.5	8.0	0.1		99.8	99.7
RpF	18.2	20.8	21.4	22.1	20.3	8.1	0.1	0.2		99.8
RpV	18.3	21.0	21.5	22.3	20.5	8.1	0.2	0.3	0.2	

Rf = *Rickettsia felis*; Rj = *Rickettsia japonica*; Rr = *Rickettsia rickettsii*; Ra = *Rickettsia australis*; Rc = *Rickettsia conorii*; Rt = *Rickettsia typhi*; RpM = *Rickettsia prowazekii* Madrid E; RpB = *Rickettsia prowazekii* Breinl; RpF = *Rickettsia prowazekii* Gv F-12; RpV = *Rickettsia prowazekii* Gv V-257.

TABLE 3

Nucleotide differences among *Rickettsia prowazekii* (*Rp*) isolates

	Nucleotide difference at position:												
	102	297	377	470	592	635	770	1783	1960	1961	2595	2665	3028	3311
*Rp*Madrid E	T	A	T	T	A	C	T	A	T	C	A	A	T	A
*Rp*Florida	G	G	–	–	–	T	–	–	–	–	–	–	G	–
*Rp*Virginia	–	G	–	C	G	T	–	G	C	A	C	C	G	–
*Rp*Breinl	–	–	G	–	–	–	C	–	–	–	–	–	G	G

(–) = No nucleotide change compared to *Rickettsia prowazekii* Madrid E strain.

fourteenth century. From there the conquistadors may have introduced epidemic typhus to the Americas. But the diseases attributed to typhus before the fifteenth century might not have been typhus at all since only in the early 1500s was the disease accurately described by Fracastoro.[25] Our hypothesis is that it was introduced into Europe during the early sixteenth century as a latent infection in a conquistador returning from the New World. Native Americans might have become infected by contact with flying squirrels, among whom *R. prowazekii* was transmitted by their own species of louse and flea in a geographically widespread, highly prevalent, enzootic cycle[6] with a highly evolved host-parasite relationship since no mortality or morbidity was observed among flying squirrels or their ectoparasites. Development of the human-louse-human cycle might have occurred after infection of louse-infested humans initially from the flying squirrel cycle. The relationship of *R. prowazekii* and human body lice (*Pediculus humanus corporis*) does not involve a high degree of adaptation of the rickettsia and the louse, since mortality in infected human body lice is 100%.

Another insect-transmitted rickettsia, *R. felis,* characterized by Azad's group and in our laboratory, has been implicated as a cause of human disease.[26] Investigations into the persistence of several foci of murine typhus in the United States revealed the presence of *R. felis* among fleas and opossums.[26,27] This led to a search for *R. felis* among murine typhus patients. Evaluation of blood samples from patients with murine typhus-like illness in Corpus Christi, Texas, by PCR amplification demonstrated that one of them contained *R. felis.*[26] In the state of Yucatán, Mexico, a rickettsiosis of the SFG clinically masquerading as dengue fever has been documented. Sera from Mexican patients suspected to have had dengue fever and later proven not to have had it were tested for antibodies to SFG and TG rickettsiae; 40% had

IgM antibodies to SFG rickettsiae.[28] Zavala and others reported three human clinical cases of rickettsial illness including a Mayan Indian woman from a rural area of Yucatán in which the etiologic agent was *R. felis* demonstrated by PCR amplification from a skin biopsy.[29]

The evolutionary relationships among members of the genus *Rickettsia* have been assessed through phylogenetic studies based on analysis of 16S rRNA and citrate synthase genes.[3,4] However, these genes have shown evolutionary proximity among the rickettsial zoonoses. The usefulness of *rompA* gene comparison for the differentiation of SFG was confirmed by Fournier,[2] who also suggested that another strongly immunogenic gene common to the SFG and TG, *rompB,* might be useful for phylogenetic inferences.

Phylogenetic analysis of the *rompB* gene of *R. felis* placed it closer to the SFG than to the TG rickettsiae. Previously, the characterization of its 16S rRNA sequence suggested a close relationship to *Rickettsia akari* and *R. australis.*[3] These data place *R. felis* as a member of the SFG in a clade with *R. akari* and *R. australis.* Similarly, phylogenetic analysis of the citrate synthase gene positioned *R. felis* within the same clade as *R. akari, R. australis,* and *Rickettsia helvetica* and identified it as belonging to the SFG rickettsiae.[3,30] Recently, the entire 5,513 base pair *rompA* gene of *R. felis* was sequenced and characterized, the strongest evidence to place *R. felis* as a member of the SFG (Bouyer and others, unpublished data), which is supported by the previous genetic analysis of the 16S rRNA and citrate synthase genes and by this report.

The *rompB* of *R. prowazekii* North American flying squirrel and European human strains differed from one another but with an overall similarity of 99.7–99.9%. Previous studies to determine the evolutionary relationships among isolates of *R. prowazekii* have focused on either biological

TABLE 4

Amino acid sequence pair distances of the rOmpB of *Rickettsia* species. Percent similarity is shown in the upper triangle and percent divergence in the lower triangle.

	Rf	Rj	Rr	Ra	Rc	Rt	RpM	RpB	RpF	RpV
Rf		84.5	82.1	81.2	84.6	71.4	71.7	71.4	71.6	71.3
Rj	15.5		89.8	79.4	92.8	65.7	66.0	65.9	65.7	65.9
Rr	17.9	10.2		77.6	95.1	65.8	65.2	65.1	64.9	65.1
Ra	18.8	20.6	22.4		79.4	66.6	65.8	65.4	65.4	65.8
Rc	15.4	7.2	4.9	20.6		67.2	67.8	67.4	67.5	67.8
Rt	28.6	34.3	34.2	33.4	32.8		86.2	86.1	86.0	86.4
RpM	28.3	34.0	34.8	34.2	32.2	13.8		99.7	99.6	99.9
RpB	28.6	34.1	34.9	34.6	32.6	13.9	0.3		99.4	99.7
RpF	28.4	34.3	35.1	34.6	32.5	14.0	0.4	0.6		99.7
RpV	28.7	34.1	34.9	34.2	32.2	13.6	0.1	0.3	0.3	

Abbreviations: Rf = *Rickettsia felis*; Rj = *Rickettsia japonica*; Rr = *Rickettsia rickettsii*; Ra = *Rickettsia australis*; Rc = *Rickettsia conorii*; Rt = *Rickettsia typhi*; RpM = *Rickettsia prowazekii* Madrid E; RpB = *Rickettsia prowazekii* Breinl; RpF = *Rickettsia prowazekii* Gv F-12; RpV = *Rickettsia prowazekii* Gv V-257.

TABLE 5
Amino acid differences of rOmpB among *Rickettsia prowazekii* (*Rp*) isolates

	Amino acid difference at position:											
	126	157	198	212	257	595	654	889	1010	1104	1123	1450
*Rp*Madrid E	V	V	T	T	V	T	S	T	Y	D	T	A
*Rp*Florida	–	–	–	I	–	–	–	–	D	–	–	–
*Rp*Virginia	–	A	A	I	–	A	Q	P	D	–	–	–
*Rp*Breinl	G	–	–	–	A	–	–	–	D	G	S	S

(–) = No amino acid change compared to *Rp* Madrid E strain.

properties or RFLP analysis.[31–35] Two of the earliest reports involved the comparative analysis of four strains of *R. prowazekii* with established strains of typhus group rickettsiae.[31,32] The first report which placed the flying squirrel isolates in the TG monitored the growth characteristics of the isolates.[31] The second report gave a detailed examination of the protein profile of four flying squirrel and two human isolates of *R. prowazekii.* These reports were able to show differences between the human and flying squirrel strains.[32] There have also been several reports by Regnery and others using restriction enzyme analysis of the genomes and RFLP analysis of selected genes of *R. prowazekii* flying squirrel isolates.[33–35] The results from these experiments showed that some PCR/RFLP patterns were identical indicating a high level of conservation within the species. It was also noted that Old World isolates of *R. prowazekii* can be distinguished from New World isolates by the banding pattern observed when the rickettsial DNA was digested with *Bam* HI or *Sac* I. Although the data gathered from the biological and RFLP analysis can be utilized to show a relationship between the isolates, there are newer mechanisms that can facilitate evolutionary analysis. DNA sequencing technologies now available can provide data that can be used to draw definite conclusions regarding the relationships among isolates of *R. prowazekii.* However, phylogenetic analysis of the *rompB* gene of the *R. prowazekii* isolates showed a very small divergence and a branching pattern that is inadequate to support our hypothesis that *R. prowazekii* originated in North

America, and then spread into the indigenous human population, and from there to Europe. The lack of significant evolutionary differences suggests that European and American strains diverged only recently. Either American strains were introduced into Europe and spread there by humans and their body lice, or the European strains were introduced into American flying squirrels, and then adapted to them over a wide geographic area in not more than a few hundred years. Molecular evolutionary clocks based on the relation between the extent of divergence in 16S rRNA and the times of divergence among eubacterial lineages, estimated a divergence rate of 1% per 50 million years.[36] This approximation was used by Stothard, who determined the amount of divergence between TG and SFG species to be approximately 1–1.3%, corresponding to 50–65 million years.[3] But this time scale cannot be applied in our analysis since it has been calibrated based on the 16S rRNA gene, an internal housekeeping gene, whereas the *rompB* gene encodes for a surface-exposed protein that is susceptible to internal and external forces.

The *rompB* gene has proven to be useful for phylogenetic comparison of the genus *Rickettsia,* but not appropriate for clarifying the evolution of the different strains of *R. prowazekii.*

Note added in press: Subsequent to the acceptance of this manuscript an article containing extensive complementary data on the phylogeny of *rompB* was published.[37]

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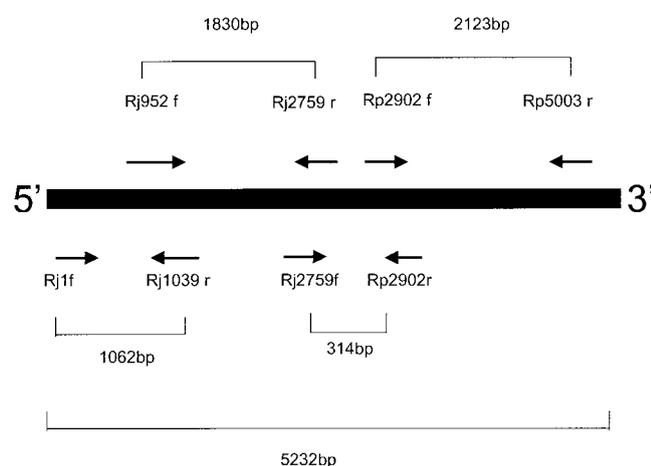

FIGURE 1. Phylogenetic tree constructed using *rompB* gene sequences of *Rickettsia* species. The tree was constructed using the maximum parsimony method. The scale indicates 100 nucleotide changes between the species. The numbers at nodes are the proportion of 1,000 bootstrap replicates that support the topology shown.

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