System. Appl. Microbiol. 18, 52–61 (1995)

Evolutionary Analysis of the Spotted Fever and Thyphus Groups of *Rickettsia* Using 16S rRNA Gene Sequences[1]

DIANE R. STOTHARD and PAUL A. FUERST*

The Department of Molecular Genetics, The Ohio State University, Columbus, OH, 43210, USA

Received October 12, 1994

Summary

The bacterial genus *Rickettsia* is traditionally divided into three biotypes, the spotted fever group (SFG), the typhus group (TG), and the scrub typhus group (STG) based on vector host and antigenic cross-reactivity. DNA sequence data were gathered from the 16S ribosomal RNA gene of several SFG and TG species. Comparative sequence analysis shows that: i) all species of *Rickettsia* are closely related, exhibiting 0.3–2.6% sequence divergence; ii) although there are identifiable clusters corresponding to the SFG and TG, species of *Rickettsia* fall into more than two distinct phylogenetic groups; iii) the tick-borne species *Rickettsia bellii* and *Rickettsia canada* diverged prior to the schism between the spotted fever and typhus groups; iv) the newly described AB bacterium is clearly a member of *Rickettsia*, but its phylogenetic placement within the genus is problematic; v) the mite-borne *Rickettsia akari*, the tick-borne *Rickettsia australis* and the recently described flea-borne ELB agent form a loose cluster that cannot be definitively associated with either the TG or the traditional SFG cluster. This latter clade may represent a unique group(s) distinct from the main cluster of spotted fever and typhus group species. The divergence of *Rickettsia* was an ancient event within the α-subclass of the proteobacteria. The sequence divergence between *Rickettsia* and *Ehrlichia*, the closest known genus to *Rickettsia*, is nearly equal to the sequence divergence between *Rickettsia* and all other α-subclass proteobacterial taxa included in the analysis. When *Rickettsia* was compared to a representative group of the α-subclass, twenty-eight nucleotide sites were identified which uniquely characterize the 16S rRNA sequences of all species of *Rickettsia*. The approximate time of divergence between the various species of *Rickettsia*, estimated from the bacterial 16S rRNA molecular clock, coincides with the approximate divergence time of the hard body ticks which are the arthropod hosts of many *Rickettsia*. Thus, the possibility of coevolution between these intracellular bacteria and their tick hosts exists.

Key words: 16S rRNA – Rickettsia – Proteobacteria – Phylogeny – Host-bacteria coevolution

Introduction

Members of the genus *Rickettsia* are gram-negative bacteria that have intimate associations with eukaryotic cells (*Weiss* and *Moulder*, 1984). They are members of a diverse family, Rickettsiaceae, which includes other intracellular forms such as *Ehrlichia*, *Wolbachia*, *Cowdria* and *Anaplasma*. They are found naturally in various arthropod hosts, including ticks, mites and insects, and can be pathogenic to man and other vertebrates. The obligate intracellular lifestyle and fastidious nature of these organisms has made them difficult to study. Originally classified with viruses because of their elusive nature, advances in both virology and rickettsiology greatly contributed to the identification of rickettsiae as bacterial organisms (*Weiss*, 1988). Evolutionary inferences, however, remained elusive.

[1] This paper is dedicated to *Willy Burdorfer* in commemoration of his receipt of an Honorary Degree, Doctor of Science from the Ohio State University, and in recognition of his lifelong contributions to rickettsiology and the microbiology of vector borne diseases.

* To whom correspondence should be addressed.

Non-standard abbreviations: SFG (spotted fever group) TG (typhus group).

The genus *Rickettsia* is divided into three biotypes on the basis of phenotypic criteria including immunological cross-reactivity and vector/host (*Weiss* and *Moulder,* 1984). The three biotypes are the spotted fever group (SFG), the typhus group (TG), and the scrub-typhus group (STG). All species of the SFG are well established in their arthropod hosts, the ixodid ticks, with the exception of *R. akari* which is mite-borne. The members of the SFG have been antigenically defined by the presence of shared group antigens rOmp A and rOmp B (*Gilmore* and *Hackstadt,* 1991; *Gilmore* et al., 1989). *Rickettsia rickettsii,* the etiological agent of Rocky Mountain spotted fever (RMSF), is the type species of the SFG and has been the most studied. Several Old World pathogens have been described, and a number of less pathogenic or non-pathogenic forms from North America have also been identified (*Dasch* and *Weiss,* 1991). For the purpose of this study, pathogenicity is considered to be related to whether a particular rickettsial species has been associated with disease in non-immunocompromised humans. The TG consists of two insect borne species, *Rickettsia prowazekii* and *Rickettsia typhi* that are similar in ecology and antigenic composition and one enigmatic tick-borne form, *Rickettsia canada.* The STG is composed of several antigenically defined mite-borne strains of the species *Rickettsia tsutsugamushi.* The lack of several major surface antigens common to SFG and TG species, including the 17kDa (*Anderson* and *Tzianabos,* 1989) and 120kDa antigens (*Gilmore* et al., 1989) and the unusual arrangement of the cell wall and plasma membrane (*Silverman* and *Wisseman,* 1978) suggest that this species is very distinct from the SFG and TG species. Consequently, it has been proposed that *R. tsutsugamushi* be placed into a different genus (*Tamura* et al., 1991).

However, there are several enigmatic species which have not been easily classified as spotted fever or typhus group species: *Rickettsia bellii* which is found in ticks but has no known natural vertebrate host (*Philip* et al., 1993); *Rickettsia akari,* found in mites (*Brettman* et al., 1981); the newly described ELB agent, found in fleas (*Adams* et al., 1990; *Williams* et al., 1992); and, the recently described ladybird beetle isolate, the AB bacterium (*Werren* et al., 1994). Using 16S and 23S rRNA genes, *R. bellii* has been identified as the product of an early divergence within this genus (*Stothard* et al., 1994). However, the phylogenetic associations of these enigmatic forms have been difficult to define due to the combination of SFG and TG characteristics they exhibit and/or the difficulty of culturing these agents, leading to the lack of phenotypic and molecular data.

Here we will present an analysis of the evolution within this genus based on a comparison of the sequences of the 16S rRNA. *Weisburg* et al. (1989) reported nearly complete sequences of the 16S rRNA gene from *R. rickettsii* and *R. typhi,* and the complete 16S rRNA sequence from *R. prowazekii.* We have recently reported the 16S rRNA gene sequences from *R. bellii, R. montana, R. rhipicephali* and the newly described *R. amblyommii* sp. nov. (*Stothard* et al., 1994; *Pretzman* et al., a, submitted). We report here six previously undetermined 16S rRNA gene sequences from *Rickettsia.* The analysis of this collection of fifteen 16S rRNA gene sequences from both SFG and TG *Rickettsia* is the first attempt to classify the majority of the described species of *Rickettsia* using DNA sequence data. The results suggest that at least four biotypes are represented in the genus *Rickettsia,* some of which are the products of divergences predating the SFG/TG schism.

While the evolutionary line that represents *Rickettsia* appears to be quite old within the α-subclass of the Proteobacteria (*Stothard* et al., 1994; *Weisburg* et al., 1989; *Werren* et al., 1994), the 16S rRNA data collected here show that all the species of *Rickettsia* form a single, closely related cluster. However, as a consequence of this low sequence variability, it also appears that the 16S rRNA gene is not a particularly informative molecule with which to analyze the detailed evolutionary relationships within this genus. Thus, we have attempted to be cautious in our analysis of the data.

Materials and Methods

Strains. All rickettsial strains were obtained from the collection of the Vector-Borne Disease Unit, Ohio Department of Health. This collection is now housed in the Department of Molecular Genetics of The Ohio State University. The strains used in this study are: *Rickettsia akari* strain "Hartford", U12458; *Rickettsia australis* strain "Phillips" (*Andrew* et al., 1946, U12459); *Rickettsia parkeri* strain "Maculatum-20" (*Bell* and *Pickens,* 1953, U12461), *Rickettsia canada,* strain McKiel (*McKiel,* et al., 1967, U15162); *Rickettsia conorii* strain ITT-586, U12460; and *Rickettsia sibirica* strain 246 (*Bell* and *Stoenner,* 1960, U12462). The sequence of the 16S rRNA gene for *Rickettsia typhi* strain "Wilmington" (*Maxcy,* 1929, U12463) used in this analysis is a sequence determined in our laboratory which clarifies the identification of ambiguous bases in the original report (*Weisburg* et al., 1989; M20499). These corrections were incorporated into our analysis accordingly. Other 16S rRNA sequences from *Rickettsia* have been reported elsewhere. (See "Additional taxa.")

Nucleic acid preparation. Rickettsiae were extracted from host cells by hostcell trypsinization, distilled water osmotic lysis, differential centrifugation of the lysate, and Sephacryl S-1000 chromatography to remove soluble contaminants. The method has been described in detail by *Pretzman* et al. (1987).

Primer design. Oligonucleotide primers for use in the polymerase chain reaction (PCR, *Saiki* et al., 1985) of the 16S rRNA gene were designed considering known conserved regions in the 16S rRNA gene of several Proteobacteria and the published 16S rRNA gene sequences for *R. rickettsii, R. prowazekii* and *R. typhi* (*Weisburg* et al., 1989). The PCR primers RA-17 and R3-17 (*Stothard* et al., 1994) were designed for amplification of the entire 16S rDNA from *Rickettsia.* In addition, several internal primers were designed for use in sequencing the 16S rRNA gene (*Stothard* et al., 1994).

Amplification. The sequences of the 16S rRNA genes were obtained from direct sequencing of PCR amplified products. The PCR conditions included 50 pmoles of each primer, 150 ng of genomic DNA, 1.25 mM dNTPs, 4 mM $MgCl_2$, and 2.5 units of Taq polymerase in a 1X reaction buffer (Perkin-Elmer Cetus). PCR was performed for 30 cycles, using the following parameters: 95 °C (1 min), 48 °C (1 min), 72 °C (1 min.). The PCR products were pipetted from beneath the mineral oil layer, the tip

was wiped clean of oil with 95% ethanol, and the PCR solution was transferred to clean tubes for sequencing.

DNA sequencing. Direct sequencing of PCR products was done using the BRL Cycle Sequencing Kit (Bethesda Research Labs, MD) as described by the manufacturer. To assure sequence fidelity, all amplified genes were sequenced from pooled multiple PCR products. In regions of variability, sequence was obtained from both strands. Consequently, an average of 80% of all sequences was obtained from both strands. In addition, the sequences were compared to the available secondary structure model for the small subunit gene (*Gutell*, 1993). Sequencing reactions were done using the dideoxy chain-termination method of *Sanger* et al. (1977) using α-^{32}P end-labeled sequencing primers. Reaction products were separated on a 6% polyacrylamide gel and visualized after an 8–24 hour exposure to X-ray film (Amersham).

Nucleotide sequence alignment. Nucleotide sequences were aligned with the assistance of the program ESEE (*Cabot* and *Beckenbach*, 1989) after identifying homologous positions on the basis of secondary structure (*Gutell*, 1993). Two alignments were generated: one included 28 taxa (see "Additional Taxa") and the other excluded all taxa except species of *Rickettsia* and the near phylogenetic taxa *Ehrlichia risticii* and *Ehrlichia sennetsu*. This alignment contained 17 taxa and included 68 sites from *Rickettsia* that had been eliminated in the 28 taxa alignment because they occurred in regions of ambiguous alignment. The length of the alignments were limited by the shortest reported sequence in the comparison, that for the ELB species. In addition, sites corresponding to PCR primers were not included in the analyses.

Additional taxa. A representative set of α-subclass proteobacterial 16S rRNA gene sequences were included for comparison with the sequences from *Rickettsia*. BLAST searches of GenBank (*Altshult* et al., 1990) using rRNA gene sequences do not suggest that other sequences are phylogenetically closer to *Rickettsia* than those included in our analysis. Nucleotide sequences for the 16S rRNA genes included in the analysis, along with GenBank accession numbers were: *Rhodobacter sphaeroides*, X53853; *Rochalimaea quintana*, M11927; *Agrobacterium tumefaciens*, X67223; *Rhizobium meliloti*, X67222; *Ehrlichia sennetsu*, M73225; *Ehrlichia risticii*, M21290; the intracellular endosymbionts of *Nasonia vitripennis*, strain B, M84687, and strain F, M84688; *Anaplasma marginale*, M60313; *Ehrlichia equi*, M73223; *Ehrlichia canis*, M73221; and *Cowdria ruminantium*, X61659. In addition, the following published sequences of *Rickettsia* were included: *R. prowazekii*, M21789; the ELB agent, L28944; the AB bacterium, U04163; *R. bellii*, U11014; *R. rickettsii*, U11021; *R. montana*, U11016; *R. rhipicephali*, U11019; *R. amblyommii* sp. nov., U11012, and the completed *R. typhi*, U12463. The 16S rRNA gene from the γ-subgroup proteobacterium, *Escherichia coli*, J01695, was used as the outgroup in the 28 taxa analysis.

Phylogenetic analysis. Once aligned, nucleotide sequences were compared and phylogenetic inferences were made using neighbor-joining (*Neighbor* with a distance matrix generated using *Dnadist* with the Kimura two-parameter correction for multiple substitutions) as implemented in Phylip Ver. 3.5 (*Felsenstein*, 1989). In addition, the data were also examined using the parsimony program *Dnapars* in Phylip. Bootstrap values for the parsimony tree were obtained from a consensus tree based on 100 randomly generated trees using *Seqboot* in Phylip. Only homologous sites (unambiguous alignment over all sequences) were compared. Consequently, 1397 sites were used in the 28 taxa comparison (which corresponds to 1344 bases from the 16S rRNA gene of *Rickettsia*), while 1412 alignable bases of the 16S rRNA gene of *Rickettsia* could be included in the 17 taxa analysis. Analyses were performed on an IRIX Indigo (Silicon Graphics, Inc.) workstation.

Results

The sequences of the 16S rRNA genes of *R. prowazekii* (*Weisburg* et al., 1989) and *R. bellii* (*Stothard* et al., 1994) were obtained from genomic clones and are each 1501 bases long. There are no insertion or deletion events that occur in the 16S rRNA gene between the defined species of *Rickettsia* which involve more than one nucleotide, with the exception of the AB bacterium sequence. Only a single amplification product resulted from use of the RA-17; R3-17 flanking primer set. We have previously reported data which shows that *Eco*RI restriction digests of a set of *Rickettsia* species, including *R. bellii*, *R. prowazekii*, and *R. rickettsii*, when probed with a 16S rDNA clone from *Rochalimaea quintana* (pMD24; gift from M. Dobson), produced patterns consistent with the presence of a single copy of the 16S rRNA gene (*Stothard* et al., 1994). Similar patterns consistent with single copies of the 16S rRNA gene were seen when we probed genomic digests (cut by either *SphI* or *Eco*RI) of *R. bellii*, *R. prowazekii* or the SFG species *R. montana*. *Pang* and *Winkler* (1993) have also shown that *R. prowazekii* contains a single copy of the 16S rRNA gene.

Two sets of analyses were done: one which included all 28 taxa and one which included species of *Rickettsia* and 2 species of *Ehrlichia*. The phylogenetic comparison of the 16S rRNA gene sequences for the 28 taxa analysis are summarized in Table 1. The phylogenetic relationships between *Rickettsia* and other members of the α-subgroup of the proteobacteria are shown in Figure 1. As earlier noted (*O'Neill* et al., 1992; *Pretzman* et al., in press) a cluster of intracellular forms, including members of *Ehrlichia, Anaplasma, Cowdria, Wolbachia*, and several unnamed endosymbionts of insects, forms a diverse sister clade to *Rickettsia*. Thus these taxa were useful to defining the placement of the root joining the *Rickettsia* species, which occurs at the branch between *R. bellii* and other *Rickettsia*. Once this root was established, *E. risticii* and *E. sennetsu* were arbitrarily used as the outgroup in the 17 taxa analysis. The 28 taxa analysis was able to verify that species of *Ehrlichia* are the closest phylogenetic relatives to *Rickettsia*. The corrected nucleotide distance between the species of *Rickettsia* is very small, being less than 2% for any of the pairwise comparisons. However, the distance between *Rickettsia* and other α-subclass proteobacteria is quite large. The average evolutionary distance between species of *Rickettsia* and the representatives of the genera *Rochalimaea, Agrobacterium, Rhizobium* and *Rhodobacter* in Table 1 is 16.7%. This value is equivalent to the average distance between *Rickettsia* and its nearest phylogenetic relatives, represented by the genus *Ehrlichia* and other groups (taxa 16–23 in Table 1), which is 17.3%. These results indicate that the genus *Rickettsia* is the product of an ancient divergence within the α-subclass of the proteobacteria.

The phylogenetic comparisons of the 16S rRNA gene sequences for the 17 taxa analysis are summarized in Table 2. The two tables are presented because they provide different levels of information about sequence divergence. Table 2 more accurately reflects the degree of intrageneric

Table 1. Evolutionary distances[a] based on the 16S rRNA gene

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1. *R. bellii*	–																											
2. *R. canada*	0.009	–																										
3. *R. typhi*	0.008	0.015	–																									
4. *R. prowazekii*	0.008	0.014	0.003	–																								
5. AB bacterium	0.009	0.016	0.015	0.015	–																							
6. ELB	0.004	0.011	0.010	0.010	0.012	–																						
7. *R. akari*	0.012	0.020	0.016	0.016	0.021	0.012	–																					
8. *R. australis*	0.006	0.014	0.011	0.011	0.015	0.004	0.012	–																				
9. *R. amblyommii*	0.008	0.015	0.012	0.012	0.012	0.008	0.018	0.011	–																			
10. *R. rhipicephali*	0.006	0.012	0.008	0.008	0.012	0.006	0.015	0.009	0.006	–																		
11. *R. montana*	0.007	0.014	0.010	0.010	0.014	0.006	0.017	0.010	0.007	0.005	–																	
12. *R. rickettsii*	0.007	0.014	0.009	0.009	0.014	0.006	0.015	0.008	0.007	0.005	0.004	–																
13. *R. parkeri*	0.008	0.015	0.012	0.012	0.015	0.008	0.019	0.011	0.010	0.008	0.006	0.005	–															
14. *R. sibirica*	0.007	0.014	0.011	0.011	0.014	0.007	0.018	0.010	0.008	0.006	0.005	0.004	0.004	–														
15. *R. conorii*	0.006	0.012	0.010	0.010	0.012	0.006	0.017	0.009	0.006	0.004	0.004	0.002	0.003	0.002	–													
16. *Ehrl. risticii*	0.177	0.181	0.180	0.178	0.185	0.180	0.179	0.181	0.184	0.179	0.179	0.178	0.178	0.177	0.177	–												
17. *Ehrl. sennetsu*	0.175	0.179	0.179	0.178	0.183	0.178	0.179	0.181	0.182	0.177	0.177	0.178	0.176	0.175	0.175	0.006	–											
18. *A. marginale*	0.167	0.170	0.165	0.165	0.173	0.170	0.172	0.171	0.169	0.167	0.164	0.164	0.165	0.166	0.164	0.156	0.154	–										
19. *Ehrl. equi*	0.170	0.172	0.169	0.168	0.175	0.170	0.172	0.172	0.171	0.169	0.166	0.166	0.169	0.168	0.166	0.155	0.153	0.022	–									
20. *C. ruminatium*	0.159	0.162	0.164	0.166	0.165	0.163	0.165	0.166	0.164	0.162	0.158	0.160	0.160	0.160	0.159	0.162	0.158	0.066	0.068	–								
21. *Ehrl. canis*	0.170	0.175	0.175	0.176	0.176	0.174	0.176	0.178	0.174	0.172	0.168	0.171	0.171	0.170	0.170	0.164	0.161	0.066	0.071	0.021	–							
22. ISym-B	0.176	0.176	0.178	0.179	0.178	0.177	0.184	0.182	0.180	0.176	0.178	0.178	0.178	0.176	0.176	0.167	0.164	0.128	0.120	0.114	0.118	–						
23. ISym-F	0.173	0.174	0.173	0.174	0.177	0.174	0.183	0.180	0.178	0.174	0.176	0.176	0.176	0.174	0.173	0.165	0.162	0.119	0.117	0.114	0.117	0.021	–					
24 *Ro. quintana*	0.158	0.165	0.164	0.164	0.164	0.158	0.164	0.161	0.163	0.162	0.160	0.161	0.164	0.162	0.161	0.205	0.203	0.178	0.170	0.182	0.186	0.193	0.188	–				
25. *Ag. tumifaciens*	0.164	0.170	0.164	0.165	0.170	0.165	0.170	0.166	0.168	0.164	0.166	0.164	0.169	0.166	0.166	0.190	0.190	0.172	0.169	0.180	0.188	0.190	0.185	0.063	–			
26. *Rz. meliloti*	0.153	0.162	0.158	0.160	0.160	0.154	0.162	0.156	0.159	0.156	0.153	0.157	0.157	0.153	0.154	0.184	0.182	0.172	0.160	0.180	0.186	0.186	0.181	0.048	0.046	–		
27. *Rh. sphaeroides*	0.180	0.186	0.182	0.182	0.185	0.184	0.184	0.183	0.184	0.182	0.180	0.182	0.183	0.182	0.181	0.202	0.200	0.182	0.174	0.185	0.190	0.193	0.194	0.118	0.116	0.098	–	
28. *Escherichia coli*	0.254	0.254	0.254	0.254	0.261	0.252	0.258	0.253	0.255	0.254	0.251	0.254	0.256	0.254	0.252	0.263	0.264	0.240	0.245	0.256	0.264	0.254	0.248	0.235	0.229	0.224	0.236	–

[a] Evolutionary distances are calculated as the average number of substitutions per sequence position adjusted as described by *Kimura* for multiple substitutions at individual positions.

Fig. 1. Phylogenetic relationship based on analysis of 16S rRNA gene sequences between the genus *Rickettsia* and other representative taxa in the α-subclass of the Proteobacteria. The tree was constructed using the neighborjoining method from the matrix of corrected nucleotide substitutions in Table 1. Nucleotide divergences between species are expressed as the sum of the horizontal distances between terminal taxa and the common ancestral node joining a pair of species. The root is placed by consideration of the *E. coli* sequence as an outlier.

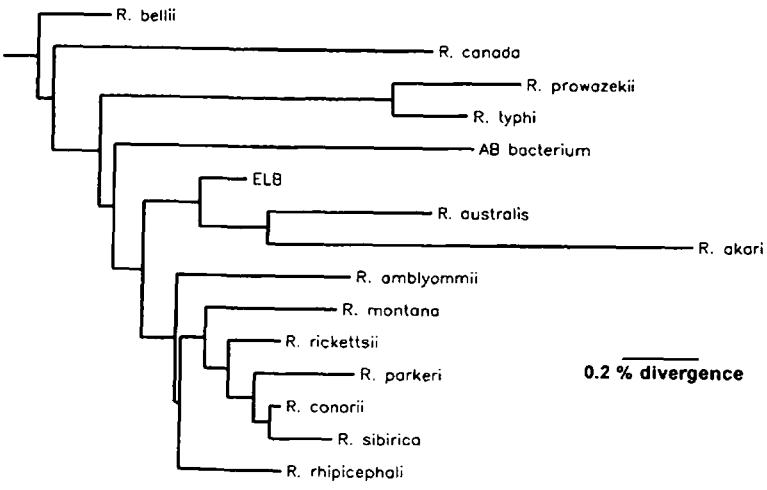

Fig. 2. Phylogenetic relationship within the genus *Rickettsia* based on analysis of 16S rRNA gene sequences from the matrix of nucletide substitutions in Table 2. The root is placed from consideration of the analysis in Figure 1. Nucleotide divergences between species are expressed as the sum of the horizontal distances between terminal taxa and the common ancestral node joining a pair of species.

Table 2. Evolutionary distances[a] between 16S rRNA gene sequences among species of *Rickettsia*

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. *R. bellii*	–																
2. *R. canada*	0.013	–															
3. AB bacterium	0.012	0.022	–														
4. *R. typhi*	0.017	0.020	0.022	–													
5. *R. prowazekii*	0.018	0.022	0.023	0.006	–												
6. ELB	0.007	0.016	0.014	0.016	0.017	–											
7. *R. akari*	0.018	0.028	0.026	0.024	0.026	0.016	–										
8. *R. australis*	0.010	0.019	0.019	0.018	0.020	0.007	0.016	–									
9. *R. amblyommii*	0.010	0.018	0.015	0.018	0.020	0.008	0.023	0.014	–								
10. *R. rhipicephali*	0.009	0.018	0.015	0.015	0.016	0.006	0.019	0.012	0.007	–							
11. *R. montana*	0.010	0.019	0.016	0.016	0.019	0.008	0.022	0.014	0.009	0.007	–						
12. *R. rickettsii*	0.009	0.018	0.015	0.015	0.016	0.006	0.018	0.010	0.008	0.006	0.006	–					
13. *R. parkeri*	0.010	0.018	0.016	0.017	0.018	0.008	0.022	0.014	0.010	0.008	0.008	0.005	–				
14. *R. sibirica*	0.010	0.019	0.016	0.017	0.018	0.008	0.022	0.014	0.009	0.007	0.007	0.004	0.005	–			
15. *R. conorii*	0.009	0.018	0.015	0.015	0.016	0.006	0.019	0.012	0.008	0.006	0.006	0.002	0.004	0.002	–		
16. *E. risticii*	0.192	0.201	0.202	0.202	0.203	0.198	0.201	0.200	0.202	0.198	0.196	0.196	0.196	0.195	0.195	–	
17. *E. sennetsu*	0.192	0.200	0.202	0.202	0.204	0.198	0.203	0.202	0.202	0.198	0.196	0.198	0.196	0.195	0.195	0.010	–

[a] The evolutionary distances are calculated as the average number of substitutions per sequence position adjusted as described by *Kimura* for multiple substitutions at individual positions.

sequence similarity between the various members of *Rickettsia,* while Table 1 provides more appropriate comparisons of homologous sites between *Rickettsia* and more distant α-subclass taxa. There were 68 nucleotide sites of the *Rickettsia* 16S rRNA gene that are not unambiguously alignable over all 28 taxa in the extended analysis and were excluded, but which can be included in the 17 taxa analysis. Among these sites, 24 were variable among the rickettsiae. Figure 2 concentrates on the branching order of the species of *Rickettsia.* This tree suggests that at least four groups exist within the genus *Rickettsia.* However, after divergence of *R. bellii* and *R. canada,* the branching order from the ancestral form cannot be determined with certainty, and occurs as a trichotomy, making evolutionary relationships within the genus difficult to determine.

At least four clusters of species appear within *Rickettsia:* two early diverging forms, *R. bellii* and *R. canada,* the TG cluster containing *R. typhi* and *R. prowazekii;* a cluster containing, *R. akari, R. australis;* and the ELB agent (the AAE clade), and a cluster which contains most of the traditional SFG species. There is also some substructure within this last group; the non-pathogenic, North American forms *R. rhipicephali, R. amblyommii,* sp. nov., and *R. montana* appear to be segregated from the pathogenic forms. The exception to this segregation of pathogens from non-pathogens is the presumed non-pathogen *R. parkeri* which will be discussed below. Both parsimony and neighbor-joining analyses were done on the 17 taxa data set along with a bootstrap analysis. The placement of the AB bacterium is problematical. It is clearly a member of *Rickettsia,* branching inside the early diverging forms *R. bellii* and *R. canada,* but not significantly associated with any of the other groups. The consensus phylogenetic trees from these analyses (not shown) indicate that *R. bellii* and *R. canada* diverged from the common rickettsial ancestor prior to the schism between a central SFG cluster and the TG. The trees obtained by the two methods differed only in the placement of the AAE clade. The consensus parsimony tree shows this group diverging prior to the SFG/TG schism while the consensus distance tree indicates they diverged from the remaining SFG species after the SFG/TG schism. However, bootstrap analysis does not support either branching order significantly more than the other.

In the 28 taxa analysis there are 512 variables sites out of the 1397 sites compared. *Rickettsia*-specific changes are found at 28 of the 512 sites (Table 3), uniquely identifying the genus compared to the other taxa in the analysis. Six of these 28 sites occur in the first 200 bases of the gene and 8 occur in a region between approximately 500 and 650 in the gene. Therefore, about half of the *Rickettsia*-specific sites occur in these two regions which include only 20% of the gene. These regions may be useful for diagnostic studies towards identifying novel rickettsia-like strains of bacteria.

Certain sequence positions also define some of the groups within *Rickettsia.* There were 82 nucleotide positions in the 16S rRNA gene which were variable in comparisons between species of *Rickettsia.* Considering only the *Rickettsia* sequences, 10 of the 82 variable sites had a

Table 3. Identification of *Rickettsia*-specific sites in the 16S rRNA gene

Site location[a]	*Rickettsia* species	Other taxa
41 (bp with 387)	T	G
99/100[b]	–	C, T, G
120 loop	A	G
153 (bp with 164)	T	A, G, C
164 (bp with 153)	A	C, T, G
380 stem	A	C, T
387 (bp with 41)	A	C
493 loop	G	T
535 loop	A	C, G
559 (bp with 597)	G	A
569 loop	G	T
585 stem	C	A, T, G
597 (bp with 559)	C	T
624 loop	G	A
659 loop	T	G
719 stem	T	G
909 (bp with 1191)	C	T
924 loop	T	C
1001 stem	A	C, G, T
1085 (bp with 1111)	T	G
1111 (bp with 1085)	A	C, T
1116 stem	A	G
1191 (bp with 909)	G	A
1227 (bp with 1232)	G	–, A, C
1232 (bp with 1227)	C	–, G, T
1253/1254[b]	–	A, C, G, T
1392 loop	T	A, C
1434 stem	A	G

[a] The numbered location of each site corresponds to its location in the *Rickettsia prowazekii* 16S rRNA gene sequence. Sites that are designated "stem" are in a stem region but are not part of a *Rickettsia*-specific base pair (bp).

[b] *Rickettsia*-specific gaps are located between the sites listed.

nucleotide difference uniquely identifying the two members of the typhus group compared to other *Rickettsia*. There are 3 sites with a nucleotide unique to the AAE clade, but there are no sites that would distinctly group either the ELB agent or *R. canada* with the TG, indicating that contrary to previous suggestions (*McKeil* et al., 1967; *Azad* et al., 1992), neither of these taxa are TG species. In addition, there are 2 sites that are unique to *R. bellii*.

Discussion

Hitherto, the species of *Rickettsia* have been grouped taxonomically mostly on the basis of morphology, pathogenicity, biochemistry, ecology and antigenic relationship (*Dasch* and *Weiss*, 1991). While all criteria are useful when studying fastidious bacteria, phenotypic characteristics can be affected by non-genetic factors, or may have evolved under strong selective pressures. Consequently, ambiguities can occur, making exact phylogenetic inferences difficult. Ribosomal RNA gene data have been gathered with the expectation that these would alleviate some of the difficulties. We have compared most of the described species of *Rickettsia* using the 16S rRNA gene. These data have revealed a more complex evolutionary history for the genus *Rickettsia* than has usually been suggested. Our results indicate that, while the genus *Rickettsia*, defined by the species in this paper, is clearly monophyletic, the tick-borne species of *Rickettsia* may be phylogenetically divided between as many as three clusters, one of which may predate the split between the traditional SFG and TG.

Analysis of the 16S rRNA gene within the genus *Rickettsia* suggests that *R. bellii* and *R. canada* represent early divergent lines within the genus *Rickettsia*, prior to the divergence of SFG and TG. Several other studies have also indicated a primitive position for the node joining *R. bellii* and the other *Rickettsia*. A limited restriction fragment length polymorphism (RFLP) analysis using three anonymous probes was done on the rickettsial genomes of 22 strains representing about 12 species (*Ralph* et al., 1990). This study was done prior to the discovery of the AB bacterium and ELB agent. The results of the RFLP study indicated that *R. bellii* is the most distant species in the genus followed by *R. akari* and *R. australis*. In addition, the results indicate a divergence of these species prior to the SFG/TG schism. *Roux* and *Raoult* (1993) have reported results from the use of pulsed-field gel electrophoresis (PFGE) to analyze RFLP variation generated by using three rare-cutting enzymes. Their analysis, however, did not include any of the TG species, *R. canada*, the AB bacterium or the ELB agent. The results, though, indicated a complex branching pattern in the SFG, with *R. bellii* being the most distantly related. Until the TG species are included in such an analysis, it is impossible to use these data to clarify if any *Rickettsia* species diverged prior to the SFG/TG schism. *Regnery* et al. (1991) used RFLP data from the citrate synthase gene and found *R. bellii* to be very divergent from the other forms compared. Similarly, *Fuerst* and *Poetter* (1991) studied DNA sequences for an anonymous region of the genome from six species and also found *R. bellii* to be very divergent.

R. canada is usually defined as a typhus group bacterium even though it is found in *Haemaphysalis* ticks. Like other typhus rickettsiae, *R. canada* grows very well in embryonated chicken eggs and dies rapidly after embryo death, and is not very cytopathic (*Ormsbee*, 1985). It also has a G+C content of 29% which is similar to the TG. However, *R. canada* is capable of cross-reacting with both SFG and TG (*Anacker* et al., 1987) and unlike other TG rickettsiae, *R. canada* can grow intranuclearly in both vertebrate and arthropod cells (*Brinton* and *Burgdorfer*, 1971; *Burgdorfer* and *Brinton*, 1970). DNA-DNA hybridization studies also indicate that *R. canada* is no more closely related to the TG species than the SFG species (*Myers* and *Wisseman*, 1981). Based on analysis of the citrate synthase gene, *R. canada* appears to be intermediate between the SFG and TG (*Regnery* et al., 1991). In addition, while no antigenically related analog of rOmp A (190 kDa antigen protein) has been identified in *R. typhi* or *R. prowazekii*, *R. canada* possess a rOmp A analog, but does not share DNA homology with the repeat region in this gene identified with the SFG rickettsiae (*Gilmore* and

Hackstadt, 1991). Therefore, it is not surprising that our analysis indicates, like *R. bellii,* that *R. canada* diverged prior to the schism between SFG and TG species.

The ladybird beetle isolate, AB bacterium, has not been well studied. Little data is available beyond the report of the 16S rRNA and 17kDa antigen gene sequences (*Werren* et al., 1994). A comparison of the 16S rRNA gene from this bacterium to several intracellular bacteria, including *R. rickettsii, R. typhi,* and *R. prowazekii,* suggested the AB bacterium belonged to a sister clade to *Rickettsia* (*Werren* et al., 1994). The 17kDa antigen gene sequence data reported in that study, however, showed that the previously undescribed species and *R. rickettsii* were more closely related than TG and SFG *Rickettsia,* suggesting a SFG origin for the form. Our results suggest a primitive position for the AB bacterium. However, support for its early divergence is weak.

R. bellii, R. canada, and the AB bacterium may not represent the only early divergent species in this genus. The biotypic composition of the AAE clade- *R. akari, R. australis* and the ELB agent- is distinct. Like *R. bellii,* the mite-borne species *R. akari* is capable of cross-reacting with both SFG and TG antisera (*Ormsbee* et al., 1978). All other traditional criteria, however, suggest that it is associated, albeit distantly, with the SFG, and specifically with *Rickettsia australis* (*Pickens* et al., 1965). *Regnery* et al. (1991) have also identified a relationship between these two species. In their study of the 190kDa antigen gene, no 190kDa gene PCR product could be amplified by the 190kDa antigen primers which successfully primed all other SFG species in their study. While *R. australis* has been grouped with the SFG, several studies have questioned this association as well. *Gilmore* et al. (1991) have determined that *R. australis* shows marked differences in its 120kDa antigen gene compared to the other SFG species. Based on their results, they suggested that *R. australis* was the most evolutionarily distant species of the SFG. The third member of this clade, the ELB agent, is found in fleas and opossums. Its discovery in murine typhus foci and the inability to amplify the 190 kDa SFG antigen gene suggested to its discoverers that it was TG in origin (*Azad* et al., 1992). However, our results do not indicate a specific TG association for this species. Rather, our analysis has identified a specific relationship between it and *R. akari* and *R. australis.* This cluster as identified by 16S rRNA sequences, does not appear to be specifically related to either SFG or TG. We suggest that the clade of *R. akari, R. australis* and the ELB agent, may represent a unique biogroup, loosely related to each other and separate from both the TG and SFG clusters.

Examination of Figure 2 and Table 2 indicates that the two typhus group species represent a highly distinct biotype, easily identifiable from the 16S rRNA gene sequence. The uniqueness of this phylogenetic divergence is emphasized by the fact that the two TG species show unique nucleotides at 10 sites, substantially more than are uniquely shared by any of the other species. The distinct nature of the TG and the very close similarity of their 16S rRNA gene sequences (Table 2) suggest that the shift of host between tick and insect occurred very early in the diversification of the genus, while the shift between insect vectors occurred much more recently.

The final group identified using the 16S rRNA gene consists of a main core of SFG species. This includes a tight cluster made up of the traditional disease-related SFG species, *R. rickettsii, R. sibirica,* and *R. conorii* as well as the apparently non-pathogenic form *R. parkeri.* The 16S rRNA gene indicates a more peripheral position for *R. montana, R. rhipicephali* and *R. amblyommii* sp. nov., North American species of unknown or unreported pathogenicity, relative to the main cluster of SFG. The primitive position of these North American species in the trees outside the Old World species suggests, contrary to some current opinion (*Marchette,* 1982), that the Old World species of the SFG may be derived from New World species.

Given the early branching within the SFG of the North American, nonpathogenic species, it is somewhat surprising to find *R. parkeri,* another North American species of unreported pathogenicity, clustering with the Old World pathogens *R. sibirica* and *R. conorii.* This placement suggests that *R. parkeri* may in fact be a cryptic pathogenic species, not yet identified with disease, or that this species is associated with a more mild or overlooked disease phenotype. In a preliminary study comparing RFLP patterns, this species was found to have the same RFLP pattern as the Hauke isolate of *R. rickettsii* (*Ralph* et al., 1990) and therefore may be mistaken for *R. rickettsii* in instances of disease. Alternatively, if *R. parkeri* is truly non-pathogenic, the interspersing of non-pathogenic and pathogenic forms suggest that changes in the ability to be pathogenic in vertebrates may have evolved more than once in this genus.

Another factor related to the phylogenetic position of *R. parkeri* is its host vector. *R. parkeri* has been isolated from primarily one species of tick, *Amblyomma maculatum* (*Parker* et al., 1939). This tick species is New World in origin. However, *Black* and *Piesman* (1994) have determined that *Amblyomma* is polyphyletic. Using small subunit rRNA sequence data from tick mitochondria they have determined that, while the New World and Old World *Amblyomma* species can be found in distinct clades, *A. maculatum* does not group in the clade with the other New World *Amblyomma* species. Therefore, the divergence pattern of the tick host *A. maculatum* parallels the divergence of the *Rickettsia* species it carries, *R. parkeri,* indicating that the segregation of *R. parkeri* from other New World species may be due to co-speciation with its host.

The various members of the genus *Rickettsia* are very closely related. While the 16S rRNA gene has proven useful for determining some relationships, it does not contain sufficient phylogenetic information necessary to completely define a statistically significant evolutionary history of this genus. This is especially obvious when analyzing the more enigmatic forms such as *R. akari, R. australis,* the ELB agent and the AB bacterium. Further work using the 23S rRNA gene and the genus-specific 17kDa antigen gene are being done to verify our analysis.

The 16S rRNA gene sequences indicate that *Rickettsia*

resulted from an ancient divergence within the α-subclass of the proteobacteria, but that the species within the genus have proliferated only recently. Given these findings, one must ask about the ecological niche of the ancestral *Rickettsia* prior to its divergence into several species. We have estimated the amount of divergence between TG and SFG species to be about 1–1.3%. Using as a first approximation, the 16S rRNA clock calibration estimated by *Wilson* et al. (1987) and the clock calibrated by *Moran* et al. (1993) based on host speciation for intracellular bacteria, and being aware of problems inherent in using a clock calibrated from a different lineage, this would correspond to 50–65 million years since these two groups diverged. The divergence between *R. bellii* and the other members would appear to be in this time range as well. The species of *Rickettsia* are confined to hematophagous arthropods, specifically several species of insects and hard body ticks (Ixodidae). The Ixodidae was previously thought to have diverged in the late Permian, 245 MYA (*Hoogstraal*, 1978). However, hard body ticks are only found more recently in the fossil record, appearing in amber from the Eocene and Oligocene, 23–56 MYA (*Sonenshine*, 1991). *Black* and *Piesman* (1994) have used a portion of the mitochondrial 16S rRNA gene from ticks to determine a molecular phylogeny for ticks. They have determined that species of *Argas*, soft body ticks, form a monophyletic and basal group to the hard body ticks. With *Argas* restricted to birds, the origin of hard body ticks would be no earlier than the late Jurrasic (140 MYA) when primitive bird fossils first appeared and no later than the late Cretaceous, early Tertiary (50–100 MYA) during the rapid radiation of bird taxa. This time scale is in agreement with the estimated divergence of *Rickettsia*, suggesting that the species of *Rickettsia* diverged at the same time that the Ixodidae diverged.

To our knowledge, only two contemporary isolates of *Rickettsia* have been obtained thus far from the argasid ticks, both of which are strains of *R. bellii* (*Philip*, 1978). Therefore, it would be logical to search for a rickettsial ancestor in the soft body ticks. If there are rickettsia-like species in these ticks, a possible evolutionary scenario would be that the ancestral *Rickettsia* species arose from forms within argasid ticks and evolved the ability to exploit the hard body ticks when they evolved in concert with mammals. Subsequently, some forms were acquired by hematophagous insects associated with mammals. This series of adaptations could eventually lead to host specificity and eventual parallel evolution or co-speciation. One possible example of co-speciation already exists within this genus, that of *R. parkeri* and its host, *A. maculatum*. Other cases may be more difficult to dissect due to the fact that i) some species of *Rickettsia* have been isolated from multiple hosts, although most, or all, seem to have a preferred host, and ii) until recently, isolation and identification of *Rickettsia* have not been done using species-specific methods. Thus, collecting evidence for co-speciation will be problematic.

Acknowledgements. We wish to thank *Chip Pretzman* for assistance in growing rickettsial cultures and isolation of DNA. Early assistance to our studies of *Rickettsia* was afforded by the Ohio Department of Health, especially the Vector Borne Disease unit and Drs. *Peg Parsons* and *Richard Berry*. Dr. *Abdu Azad* was very helpful in providing DNA from *R. canada* and furnishing information about his studies of the ELB agent. Dr. *David Walker* provided useful insights concerning an earlier version of the manuscript. We also wish to thank Dr. *Rebecca Gast* for assistance in using the IRIX Indigo computer system.

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Paul A. Fuerst, Department of Molecular Genetics, The Ohio State University, 484 West 12th Avenue, Columbus, OH 43210, Phone: 614-292-6403; FAX: 614-292-4466; e-mail: pfuerst@magnus.acs.ohio-state.edu