Phylogeny of *Rickettsia* spp. inferred by comparing sequences of 'gene D', which encodes an intracytoplasmic protein

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'Gene D' is the PS120-protein-encoding gene, first described in *Rickettsia conorii* and *Rickettsia japonica*. Sequence analysis of a 3030 bp fragment of 'gene D' in 24 representatives of the genus *Rickettsia* was carried out to complete phylogenetic analyses previously inferred by comparison of gene sequences encoding citrate synthase, 17 kDa antigen and rOmpA and rOmpB. The phylogenetic relationships between rickettsiae were inferred from the comparison of both the gene and the derived protein sequences, using the parsimony, neighbour-joining and maximum-likelihood methods. Five distinct groups of rickettsiae were identified. These were: the *Rickettsia massiliae* group, including *R. massiliae*, Bar 29, *Rickettsia rhipicephali* and *Rickettsia aeschlimannii*; the *Rickettsia rickettsii* group containing *Rickettsia sibirica*, '*Rickettsia mongolotimonae*', *Rickettsia parkeri*, strain S, *Rickettsia africae*, the *R. conorii* complex, *Rickettsia slovaca*, *Rickettsia honei*, *R. rickettsii*, *R. japonica* and *Rickettsia montanensis*; the group currently containing only *Rickettsia helvetica*; the *Rickettsia akari* group including *Rickettsia australis*, *R. akari* and the ELB agent; *Rickettsia prowazekii* and *Rickettsia typhi* clustered in the typhus group. As significant bootstrap values were obtained for most of the nodes, sequence comparison of 'gene D' should be considered as a complementary approach in phylogenetic studies of rickettsiae.

Keywords: gene D, *Rickettsia*, phylogenetic analysis

INTRODUCTION

Rickettsia are obligate intracellular Gram-negative bacteria and although arthropods are more often their main reservoir hosts, people and other vertebrates may also be infected. Comparative microimmunofluorescence (MIF) testing with polyclonal mouse sera (Philip *et al.*, 1978; Beati *et al.*, 1994; Ereemeeva *et al.*, 1995) has long been the reference method for the identification of rickettsiae but genotypic identification is now used more frequently. Early reports showed interspecies differences using RFLP analysis of PCR-amplified DNA (Regnery *et al.*, 1991; Ereemeeva *et al.*, 1994). Later, significant differences in the 16S rRNA

gene sequences of members of the genus *Rickettsia* resulted in its division into two genera. The first one was the newly created genus *Orientia* (Tamura *et al.*, 1995) and the other was the genus *Rickettsia*, which was itself subdivided in two groups. The typhus group (TG) included *Rickettsia prowazekii*, *Rickettsia typhi* and *Rickettsia canadensis* while the spotted fever group (SFG) included all other rickettsiae. Recent phylogenetic studies of rickettsiae using genes encoding 16S rRNA (Stothard & Fuerst, 1995; Roux & Raoult, 1995), citrate synthase (*gltA*) (Roux *et al.*, 1997), 17 kDa antigen (Stenos *et al.*, 1998; Anderson *et al.*, 1988), rOmpA (Fournier *et al.*, 1998) and rOmpB (Roux & Raoult, 2000) have suggested a different organization for the genus. Several distinct clusters of organisms were shown to occur in the SFG, indicating the heterogeneity of the genus. Moreover, *R. canadensis* and *Rickettsia bellii* were shown to be sufficiently distinct to warrant their removal from the TG and SFG, respectively. As new rickettsiae continue to be identified, it is important to select informative

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Abbreviations: SFG, spotted fever group; TG, typhus group.

The GenBank accession numbers for the nucleotide sequences reported in this paper are given in Methods.

Table 1. List of the strains included in the study

Rickettsia	Strain	Size of the sequenced gene fragment (bp)
<i>R. aeschlimannii</i>	MC16 ^T	3010
<i>R. africae</i>	EFS-5	3034
Astrakhan fever rickettsia	A-167	3028
<i>R. australis</i>	Phillips	2999
<i>R. akari</i>	MK, ATCC VR-148 ^T	2995
Bar 29	Bar 29	3034
<i>R. conorii</i>	Moroccan, ATCC VR-141	3028
<i>R. conorii</i>	Indian tick typhus rickettsia, ATCC VR-597	3028
ELB bacterium	URRWX Cal ₂	2991
<i>R. helvetica</i>	C9P9	3028
<i>R. honei</i>	Thai tick typhus rickettsia, ATCC VR-599 ^T	3034
Israeli tick typhus rickettsia	ISTT CDC1	3028
<i>R. japonica</i>	YM	3016
<i>R. massiliae</i>	Mt1 ^T	3031
' <i>R. mongolotimonae</i> '	HA-91	3034
<i>R. montanensis</i>	M/5-6	3028
<i>R. parkeri</i>	Maculatum 20	3031
<i>R. prowazekii</i>	Brein1, ATCC VR-142 ^T	3018
<i>R. rhipicephali</i>	3-7-6	3040
<i>R. rickettsii</i>	R (Bitter root), ATCC VR-891 ^T	3022
Strain S	Strain S	3034
<i>R. sibirica</i>	246, ATCC VR-151 ^T	2974
<i>R. slovaca</i>	13-B	3037
<i>R. typhi</i>	Wilmington, ATCC VR-144 ^T	3022

gene sequences to establish phylogenetic analysis and classification of these organisms.

'Gene D' is a recently described gene in rickettsiae and its potential for facilitating phylogenetic analysis of these bacteria has yet to be considered. It was first identified by Schuenke & Walker (1994) from three overlapping constructs derived from genomic *Rickettsia conorii* DNA libraries. Sequencing revealed it to be a 3065 nucleotide open reading frame encoding a protein which was recognized in humoral and cell-mediated immune responses and was thus suspected of playing a role in protective immunity (Schuenke & Walker, 1994). The sequencing data also showed that 'gene D' had no significant homology with any previously sequenced genes. Later, a major part of the corresponding 'gene D' of *Rickettsia japonica* was cloned and recombinant protein was expressed in *Escherichia coli* (Uchiyama *et al.*, 1996). Antisera against the recombinant protein were found to react with the heat-stable 'PS120' proteins of *R. japonica* (Uchiyama *et al.*, 1996). These proteins are distinct from the rOmpB outer-membrane protein (Carl *et al.*, 1990) and, by immunoelectron microscopy, are found outside the electron-lucent nucleoid-like region in the cytoplasm of *R. japonica* (Schuenke & Walker, 1994; Uchiyama, 1997).

In this report, we describe the sequencing of 'gene D' in 24 representatives of the genus *Rickettsia* and the

phylogenetic data obtained by comparison of these sequences.

METHODS

Rickettsial strains. The strains used are listed in Table 1.

Rickettsial cultivation. The SFG and TG rickettsiae were grown on Vero cell monolayers as previously described (Roux *et al.*, 1997). When Gimenez staining showed the cells to be heavily infected, they were harvested and centrifuged at 12000 g for 10 min, resuspended in medium and stored at -70 °C until purification of nucleic acid was carried out.

Nucleic acid purification, PCR amplification and sequencing reactions. Genomic DNA was extracted using Qiagen columns (QIAamp tissue kit; Qiagen) according to the manufacturer's instructions. PCR amplification and the sequencing reactions were performed using the oligonucleotide primers listed in Table 2. The first 10 primers in the table were identified from sequence data on *R. conorii* (GenBank accession no. U01133) and used to amplify 'gene D' in five fragments (from I to V), each of a size suitable for subsequent sequencing. These primers amplified the gene in all the rickettsiae studied apart from *Rickettsia akari*, *Rickettsia australis*, ELB agent, *R. prowazekii* and *R. typhi* (see later). Specific primers D3064RICr and D3073JAPr were selected to amplify the 3' end of the gene in *Rickettsia rickettsii* and *R. japonica*. A mutation in the 19th position of the primer D2338f explained the necessity to modify the sequence of this primer for *Rickettsia massiliae*, Bar 29 and *Rickettsia rhipicephali*. The specific primers D1246HELf

Table 2. Oligonucleotide primers used for PCR amplification and sequencing of rickettsial species

Primer	Nucleotide sequence 5' → 3'	Position in the gene	Primer used for:	Fragment
D1f	ATG AGT AAA GAC GGT AAC CT	1–20	Most of the rickettsiae	I
D928r	AAG CTA TTG CGT CAT CTC CG	928–907	Most of the rickettsiae	I
D767f	CGA TGG TAG CAT TAA AAG CT	767–786	Most of the rickettsiae	I
D1390r	CTT GCT TTT CAG CAA TAT CAC	1390–1370	Most of the rickettsiae	II
D1219f	CCA AAT CTT CTT AAT ACA GC	1219–1237	Most of the rickettsiae	II
D1876r	TAG TTT GTT CTG CCA TAA TC	1876–1857	Most of the rickettsiae	III
D1738f	GTA TCT GAA TTA AGC AAT GCG	1738–1758	Most of the rickettsiae	III
D2482r	CTA TAA CAG GAT TAA CAG CG	2482–2463	Most of the rickettsiae	IV
D2338f	GAT GCA GCG AGT GAG GCA GC	2338–2357	Most of the rickettsiae	IV
D3069r	TCA GCG TTG TGG AGG GGAA G	3069–3050	Most of the rickettsiae	V
D547r	TAA ATC CAC CAG GGT ATC CA	547–528	Most of the rickettsiae	V
D2762f	CTA AAG AGG TAC GTT TAC AAA C	2762–2783	Most of the rickettsiae	V
D3064RICr	GTT GTG GAG GGG AAG ACT CTC	3064–3044	<i>R. rickettsii</i>	V
D3073JAPr	TTG ATC AGC GTT GTG GAG GG	3073–3054	<i>R. japonica</i>	V
D2338MBRf	GAT GCA GCG AGT GAG GCA AC	2357–2338	<i>R. massiliae</i> , Bar 29, <i>R. rhipicephali</i>	V
D1246HELf	TTA TCA GGC AGC ATG CAA GA	1246–1265	<i>R. helvetica</i>	III
D1862HELr	GTA ATC TTA TCT AAA TGC TGC	1862–1842	<i>R. helvetica</i>	III
D1652SIBf	CAA TTG ACG GTA TTA AGG ATG	1652–1672	<i>R. sibirica</i>	IV
D727CONf	CCT ATA AAA CTT GAT AAA GCC	727–747	<i>R. akari</i> group and TG	
D791CONr	CCA TCA GCT TTT AAT GCT AC	791–772	<i>R. akari</i> group and TG	
D1605CONf	AGT AGA TGT CGG TTT AAG T	1605–1623	<i>R. akari</i> group and TG	
D1835CONr	AAT TGT TGT TGT KCC GGA CT	1816–1835	<i>R. akari</i> group and TG	
DGW238AUSr	GGT CTG ATG TCT GAC CGT CA	238–219	<i>R. australis</i>	
DM67AAf	TTA CTT ACA GTT TGT GCT AA	M67–M47	<i>R. australis</i> , <i>R. akari</i>	
D328AAEf	GAC CGT GAT TTA GCT GAA CA	328–347	<i>R. australis</i> , <i>R. akari</i> , ELB bacterium	
D347AAEr	TGT TCA GCT AAA TCA CGG TA	347–328	<i>R. australis</i> , <i>R. akari</i> , ELB bacterium	
D1204AAEf	TCG CAA CAA GTG AAT CCA AA	1204–1223	<i>R. australis</i> , <i>R. akari</i> , ELB bacterium	
D1357AAEr	TAA GAA TTG CNG TAG CTG CT	1357–1338	<i>R. australis</i> , <i>R. akari</i> , ELB bacterium	
DGW1967AUSf	CAA AGG TTT TAG ATR GTC CG	1967–1986	<i>R. australis</i>	
DGW2344AUSf	GCG AGT GAG GYA GCT TTA GAT	2344–2364	<i>R. australis</i>	
DGW2594AUSf	ATT TTC GTG CTA TGC TYT CT	2594–2613	<i>R. australis</i>	
D2996AKAr	CTT GCA TTC ATT AAT GCT CC	2996–2977	<i>R. akari</i>	
D2077AAEr	CAA TGT CTG CTT TAT CTT G	2077–2058	<i>R. australis</i> , <i>R. akari</i> , ELB bacterium	
D2406AAEr	ATT CTG TCC TTT TAA CCC TT	2406–2387	<i>R. australis</i> , <i>R. akari</i> , ELB bacterium	
D2684AAEr	TTT ACC AGC TCA ACT ACT TT	2684–2665	<i>R. australis</i> , <i>R. akari</i> , ELB bacterium	
D51ELBf	TAA GGA ATA TAC AGA AGA A	51–69	ELB bacterium	
D3022ELBr	TTA AAT TTT CCG ACT GAA TG	3022–3003	ELB bacterium	
DGW321TPf	TAA TAC AAA TCC AGA ACT MGC TG	321–343	<i>R. prowazekii</i> , <i>R. typhi</i>	
DGW343TPr	CAG CKA GTT CTG GAT TTG TAT TA	343–321	<i>R. prowazekii</i> , <i>R. typhi</i>	
DGW1141TPf	CAA GTG CCA CCA ATC ACT ACG	1141–1161	<i>R. prowazekii</i> , <i>R. typhi</i>	
DGW1161TPr	CGT AGT GAT TGG TGG CAC TTG	1161–1141	<i>R. prowazekii</i> , <i>R. typhi</i>	
DGW2069TPf	CAG AGA TTG TTA AAG GTA TAG G	2069–2090	<i>R. prowazekii</i> , <i>R. typhi</i>	
DGW2090TPr	CCT ATA CCT TTA ACA ATC TCT G	2090–2069	<i>R. prowazekii</i> , <i>R. typhi</i>	
DGW2444TPf	AAA CGC AAG ATG TWG CTA ATG	2444–2464	<i>R. prowazekii</i> , <i>R. typhi</i>	
DGW2464TPr	CAT TAG CWA CAT CTT GCG TTT	2464–2444	<i>R. prowazekii</i> , <i>R. typhi</i>	

and D1862HELr were used to amplify fragment III of the *Rickettsia helvetica* gene and primer D1652SIBf was used in place of D1738f for the amplification of *Rickettsia sibirica*.

Each PCR was carried out in a Peltier model PTC-200 thermal cycler (MJ Research). Five microlitres of the purified DNA was amplified in a 50 µl reaction mixture containing 25 pmol of each primer, 200 mM (each) dATP, dCTP, dGTP and dTTP (Gibco-BRL Life Technologies), 1 U ELONGASE (Gibco), 2 µl buffer A and 8 µl buffer B. Amplification was carried out with an initial 3 min denaturation at 95 °C followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 68 °C for 1 min 30 s. The amplification was completed by holding the reaction mixture for 7 min at 68 °C to allow complete extension of the PCR products.

PCR products were purified using a QIAquick Spin PCR purification kit (Qiagen) as described by the manufacturer. Sequencing reactions were carried out using a DNA sequencing kit (dRhodamine Terminator cycle sequencing ready reaction with Amplitaq Polymerase FS) (PE Applied Biosystems) as described by the manufacturer. Sequence products were purified and resolved on 5% (w/v) polyacrylamide gels (Long Ranger Singel packs, Type 377-36 cm WTR) (Tebu) and electrophoresis was performed with the ABI PRISM 377 DNA sequencer (Perkin Elmer). The same primers as those used for PCR amplification were used for the sequencing reactions. Primers D547r and D2762f were used to confirm the sequence of the 5' and 3' end of the gene, respectively.

No PCR amplification was obtained with the primer pairs described above for the other rickettsiae. The rickettsial sequences obtained were aligned and two consensus primers were selected, D727CONf and D1835CONr. PCR amplification was obtained for *R. akari*, *R. australis* and the ELB bacterium. An improved PCR method for walking in uncloned genomic DNA, using the Universal Genome Walker kit (CLONTECH Laboratories) (Siebert *et al.*, 1995), had to be used for the determination of the sequence of *R. australis*. Primers D791CONr and DGW238AUSr were used to determine the 5' end of the 'gene D' sequence, and the primers D1605CONf, DGW1967AUSf, DGW2344AUSf and DGW2594f to determine the 3' end of the sequence. Primers DM67AAf and D791CONr and primers D1605CONf and D2996AKAr were used to amplify the 5' and 3' ends of the *R. akari* gene, respectively, while primers D51ELBf and D791CONr and primers D1605CONf and D3022ELBr were used to amplify the 5' and 3' ends of the ELB bacterium gene, respectively. Primers D328AAEf, D347AAEr, D1204AAEf, D1357AAEr, D2077AAEr, D2406AAEr and D2684AAEr were determined to confirm the sequence of 'gene D' of *R. akari*, *R. australis* and the ELB bacterium.

Amplification of *R. prowazekii* and *R. typhi* was carried out with primers D727CONf and D1835CONr and primers D1141TPf and D1161TPr. The genome walker approach was then used. Primers D791CONr, DGW321TPf and DGW343TPr were incorporated in the PCR amplification and sequencing reactions to determine the 5' end sequences while primers DGW2069TPf, DGW2090TPr, DGW2444TPf and DGW2464TPr were used to determine the 3' end sequences.

Data analysis. The 'gene D' sequences were translated into protein sequences using PC/GENE software (IntelliGenetics). The nucleic and amino acid sequences were aligned using the multisequence alignment program CLUSTAL within the

BISANCE environment (Dessen *et al.*, 1990). The percentages of similarity were determined using the PC/GENE software package. The phylogenetic relationships between the representatives of the genus *Rickettsia* studied were determined using the 3.4 version of the PHYLIP software package (Felsenstein, 1989). Distance matrices generated by DNADIST and PROTDIST were determined under the assumptions of Kimura (1980). These matrices were used to elaborate dendrograms using the neighbour-joining method (Saitou & Nei, 1987). The data were also examined by using parsimony and maximum-likelihood methods (DNAPARS and PROTPARS, and DNAML in PHYLIP). A bootstrap analysis was performed to investigate the stability of the resulting trees. Bootstrap values were obtained for a consensus tree based on 100 randomly generated trees by using SEQBOOT and CONSENSE in the same package.

Nucleotide sequence accession number. The GenBank accession numbers for the nucleotide sequence data reported in this paper are as follows: *Rickettsia aeschlimannii*, AF163005; *Rickettsia africae*, AF151724; Astrakhan fever rickettsia, AF163007; *R. australis*, AF187982; *R. akari*, AF213016; Bar 29, AF155056; ELB bacterium, AF196973; *R. helvetica*, AF163009; Israeli tick typhus rickettsia, AF155058; *R. japonica*, AF155055; *R. massiliae*, AF163003; '*Rickettsia mongolotimonae*', AF151725; *Rickettsia montanensis*, AF163002; *Rickettsia parkeri*, AF155059; *R. prowazekii*, AF200340; *R. rhipicephali*, AF155053; *R. rickettsii*, AF163000; strain S, AF163001; *R. conorii* strain Moroccan, AF163008; *R. sibirica*, AF155057; *Rickettsia slovaca*, AF155054; *Rickettsia honei*, AF163004; *R. typhi*, AF188482; *R. conorii* strain Indian tick typhus rickettsia, AF163005.

RESULTS

PCR amplification and sequencing

'Gene D' fragments were amplified from all the strains of *Rickettsia* studied except *R. bellii* (strain 369L42-1) and *R. canadensis* (strain 2678). For each species the sequence of both DNA strands was determined at least twice. The 'gene D' was sequenced between bases 21 and 3050 for most of the species with respect to the open reading frame of the published sequence of *R. conorii*. Identical sequences were obtained to those already published for *R. conorii* (GenBank accession no. RCU01133), *R. japonica* (AB003696) and *R. prowazekii* (AJ235272). Minor differences in the sequences of the Indian tick typhus and Moroccan strains of *R. conorii* were obtained. The highest percentage of similarity between different species was obtained for the Israeli tick typhus rickettsia and the Astrakhan fever rickettsia (99.7% for DNA sequences and 99.5% for protein sequences) and the lowest similarity was between *R. prowazekii* and *R. akari* (76.3% for DNA sequences and 59.7% for protein sequences).

Phylogeny inference

Nucleotidic 'gene D' sequences were considered from base 53 to base 3006 to infer phylogenetic analysis. The dendrograms obtained with the three different tree-building analysis methods used showed similar

Fig. 1. Phylogenetic tree of the bacteria belonging to the genus *Rickettsia* inferred from comparison of the 'gene D' sequences. The evolutionary distance values were determined by the method of Kimura, and the tree was constructed by the neighbour-joining method. The numbers at nodes are proportions of 100 bootstrap resamplings that support the topology shown.

patterns. The two representatives of the TG, *R. prowazekii* and *R. typhi*, clustered together. *R. akari*, *R. australis* and the ELB bacterium clustered together and formed the *R. akari* group. Another distinct cluster contained *R. massiliae*, Bar 29, *R. rhipicephali* and *R. aeschlimannii*. *R. helvetica* was alone on a separate branch between the TG and the *R. akari* group in the parsimony and maximum-likelihood analyses and between the *R. akari* and *R. massiliae* groups in the distance matrices analysis. The *R. rickettsii* group was a large cluster containing 14 representatives: *R. africae*, strain S, *R. parkeri*, *R. sibirica*, '*R. mongolotimonae*', the *R. conorii* complex (*R. conorii* strains, Astrakhan fever rickettsia and Israeli tick typhus rickettsia), *R. slovaca*, *R. rickettsii*, *R. honei*, *R. montanensis* and *R. japonica*. In this group, the phylogenetic organization was the same when the neighbour-joining and the maximum-likelihood analyses were used (Fig. 1). Differences were, however, noted in the parsimony analysis. *R. montanensis* diverged before *R. japonica* and *R. sibirica* clustered with *R. rickettsii* between the *R. conorii* complex and another group including *R. africae*, strain S, *R. parkeri* and '*R. mongolotimonae*'. For a particular method of analysis, the tree inferred from the protein sequences was practically the same as the one obtained from the nucleic acid sequences. However, in the parsimony analysis, *R. aeschlimannii* clustered with *R. montanensis* between the *R. massiliae* group and the *R. rickettsii* group and in the neighbour-joining analysis *R. slovaca* clustered with the *R. conorii* complex.

Significant bootstrap values (> 70%) were obtained for most of the nodes. In the neighbour-joining analysis when phylogeny was inferred from protein sequences, the node where *R. slovaca* branched was not well-supported. The same observation was also noted for the node where *R. massiliae* and Bar 29 clustered and the node where *R. aeschlimannii* branched when phylogeny was inferred from DNA sequences and for the nodes where *R. parkeri* and *R. montanensis* diverged in phylogenies inferred from both DNA and protein sequences. In the parsimony analysis when phylogenies were inferred from both DNA and protein sequence comparison, non-significant values were obtained for the nodes where *R. africae*, *R. parkeri*, strain S, '*R. mongolotimonae*', *R. rickettsii* and *R. sibirica* branched. For phylogeny inferred from comparison of protein sequences no significant value was obtained for the node where *R. montanensis* and *R. aeschlimannii* clustered. In the maximum-likelihood analysis when phylogeny was inferred from DNA sequence comparison, non-significant values were obtained for the nodes where *R. parkeri* and *R. montanensis* branched.

DISCUSSION

It has been suggested that the method of choice to establish bacterial phylogeny is the comparison of different gene sequences (Olsen & Woese, 1993). So, the division of rickettsial species into two phyla, the SFG and TG, is not supported by genomic data. The

Fig. 2. Schematic representation of the dendrograms obtained by rickettsial sequence comparison of different genes. The phylogenetic organization is that obtained when the evolutionary distance values were determined by the method of Kimura and the tree was constructed by the neighbour-joining method. RRG, *Rickettsia rickettsii* group; RJ, *R. japonica*; RM, *R. montanensis*; RMG, *R. massiliae* group; RH, *R. helvetica*; ELB, ELB bacterium; RAG, *R. akari* group; TG, typhus group; RC, *R. canadensis*; RB, *R. bellii*.

16S rRNA gene is highly conserved in bacteria and phylogenetic analysis inferred from its sequence comparison can be a useful phylogenetic tool although it cannot be used to reliably classify *Rickettsia* spp. (Roux & Raoult, 1995). For enteric bacteria, phylogeny studies based on *rpoB* gene sequences have been shown to be more reliable than those based on the 16S rRNA gene (Mollet *et al.*, 1997). In the SFG rickettsiae, bacteria included in the *R. massiliae* group, as defined on the basis of *ompB* gene sequence comparison (*R. montanensis*, *R. aeschlimannii*, *R. massiliae*, Bar 29 and *R. rhipicephali*), were shown to be the only rifampin-resistant rickettsiae (Rolain *et al.*, 1998). The outer-membrane protein genes *ompA* and *ompB* were the more reliable tools to study the phylogeny of *Rickettsia* species.

In our experiments, we investigated the comparison of 'gene D' sequences as a tool to infer phylogeny of rickettsiae. The major discrepancy between our results and those previously reported for the *gltA*, *ompA* and *ompB* genes was that *R. montanensis* clustered in the *R. rickettsii* group. Our study confirmed the presence of a TG with only two members, *R. typhi* and *R. prowazekii*, the vectors of which are fleas and body lice, respectively.

R. helvetica was found alone on a separate branch but its phylogenetic position was not stable and varied according to the phylogenetic analysis method used.

The *R. akari* group comprised three representatives: *R. australis*, transmitted by ticks; *R. akari*, transmitted by mites; and the flea-borne rickettsia ELB agent also named '*Rickettsia felis*'. Previous studies based on sequence comparisons of genes encoding 16S rDNA and the 17 kDa protein suggested that the ELB agent was within the TG (Radulovic *et al.*, 1995; Higgins *et al.*, 1996; Schiefer *et al.*, 1994) but our results are in agreement with those obtained from the *gltA* gene sequence comparison. The phylogenetic organization of the *R. massiliae* group was not as stable as those determined from *ompA*, *ompB* and *gltA* gene sequence comparison. In fact, significant bootstrap values were not always obtained and the inclusion of *R. aeschlimannii* in this group depended on the method of analysis used. However, *R. montanensis* was always excluded from this group.

In the *R. rickettsii* group, the *R. conorii* complex organization and the cluster *R. africae*/strain S were well established. The presence of the other representatives (*R. parkeri*, '*R. mongolotimonae*', *R. sibirica*, *R. slovaca*, *R. rickettsii*, *R. honei*, *R. japonica* and *R. montanensis*) in the *R. rickettsii* group was constant but comparison of their phylogenetic relationships was dependent on the method used.

We were not able to amplify 'gene D' in *R. canadensis* and *R. bellii*, which have previously been shown to cluster with the AB bacterium (Werren *et al.*, 1994).

and with the PTB bacterium (Davis *et al.*, 1998), respectively, when comparing 16S rDNA sequences (Roux & Raoult, 1995; Stothard & Fuerst, 1995). Comparison of the gene sequences encoding citrate synthase has also indicated that these two rickettsiae may be excluded from both the TG and SFG rickettsiae (Roux *et al.*, 1997).

In attempting to establish a phylogeny of the *Rickettsia*, combination of the data obtained from different studies has indicated the need to reconsider the classification of these bacteria into more than two groups. The organization of the different clusters obtained from rickettsial sequence comparison of different genes is schematized in Fig. 2. Thirteen representatives of the genus *Rickettsia* are included in the *R. rickettsii* group: *R. conorii* strains, Astrakhan fever rickettsia, Israeli tick typhus rickettsia, *R. sibirica*, '*R. mongolotimonae*', *R. africae*, strain S, *R. parkeri*, *R. slovaca*, *R. rickettsii*, *R. honei* and *R. japonica*. A different arrangement inside this group was obtained when phylogeny was inferred from a comparison of different gene sequences. However, those obtained from *ompA* and *ompB* analyses, which were identical, could correspond more closely to the true evolution of this group of the *Rickettsia* as significant bootstrap values were obtained for all the nodes in the three analysis methods (neighbour-joining, parsimony and maximum-likelihood) from both DNA and protein sequences. Moreover, where clustering differed between *gltA* and 'gene D' sequence comparisons, bootstrap values at the relevant nodes were not significant.

The phylogenetic position of *R. montanensis* is ambiguous. If we consider *gltA* and *ompA* analyses, it was outside the group including *R. massiliae*, Bar 29, *R. rhipicephali* and *R. aeschlimannii*. The bootstrap values at the corresponding nodes were significant, being 100% in both reconstructions. However, if we consider *ompB* analysis, *R. montanensis* clustered inside this group, with a significant bootstrap value of 88%. In 'gene D' analysis it clustered with the *R. rickettsii* group but the bootstrap value was not significant (51%). More information is required to precisely determine the phylogenetic position of *R. montanensis*. However, the grouping of *R. massiliae*, Bar 29, *R. rhipicephali* and *R. aeschlimannii* is well established in the four analyses.

R. helvetica has followed a particular evolution and was always found alone on a separate branch, more often between the ELB bacterium and the group including *R. massiliae*. The phylogenetic position of the ELB bacterium is also ambiguous as either it clustered with the *R. akari* group (*R. akari* and *R. australis*) (*gltA* and 'gene D' analyses) or it was alone on a separate branch (*ompB* analysis). It seems that its preferential position is to cluster with the *R. akari* group as in the two cases bootstrap values are 100% compared with 73% in the *ompB* analysis, which is just above the limits of significance. *R. akari* and *R.*

australis always clustered together, as did *R. typhi* and *R. prowazekii*, which formed the TG.

We suggest that *R. canadensis*, *R. bellii* and the AB bacterium were the first representatives of the genus *Rickettsia* to diverge from the common ancestor of this group of bacteria. Few sequences are available for these rickettsiae as often it was impossible to amplify DNA from the genes when amplification was carried out with consensus primers due to their major genomic divergence. More investigation will be particularly necessary to establish the phylogenetic relationships in this group.

Phylogenetic analyses of rickettsial species have advanced considerably during the last few years particularly due to the introduction of the molecular biology methodology. Nevertheless ambiguities remain. In the future, further information will be necessary to improve the phylogeny and establish a taxonomy of the genus *Rickettsia*.

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