Rickettsiae phylogeny: a multigenic approach

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The development of molecular taxonomic methods has provided a large amount of data in the reorganization of *Rickettsiae* taxonomy. Nevertheless, phylogenetic relationships among some groups and species delimitation remain unclear. To clarify rickettsial phylogeny, a multigenic approach was used for the first time for the genus *Rickettsia*, based on simultaneous analyses of eight loci: *atpA*, *recA*, *virB4*, *dnaA*, *dnaK*, *rrl-rrf* internal transcribed spacer, *ompA* and *gltA*. Concatenation of different nucleotide sequences resulted in an improvement in phylogenetic resolution when compared to single gene data. This multigenic approach has enabled the differentiation of many groups, including the spotted fever group which includes a great number of closely related species. The reliability of some previously recognized groups was evaluated.

INTRODUCTION

The genus *Rickettsia* belongs to the *Alphaproteobacteria* and represents a group of obligate intracellular endosymbionts of eukaryotic cells (Weiss & Dasch, 1991). The genus subdivides into the conventionally well defined typhus group (TG) and spotted fever group (SFG), based mainly on phenotypic and serological features. The genus *Rickettsia* also includes several marginal species, such as *Rickettsia canadensis* and *Rickettsia bellii*, the most divergent species within the *Rickettsiae* (Stothard *et al.*, 1994). The recently sequenced *R. bellii* genome was shown to be larger than the other rickettsial genomes sequenced so far and exhibits little colinearity with any of them, suggesting that this species may have retained several ancestral features lost in other lineages in the course of evolution (Ogata *et al.*, 2006).

The TG includes *Rickettsia prowazekii* and *Rickettsia typhi*, which are the aetiological agents of insect-borne epidemic and endemic typhus, respectively. The SFG consists of several species, some of which are the causative agents of well known tick-borne diseases, such as Rocky Mountain spotted fever (*Rickettsia rickettsii*) and Mediterranean spotted fever (*Rickettsia conorii*) (Raoult & Roux, 1997).

Rickettsiae phylogeny has been addressed by sequence analyses of certain genes, mostly those belonging to the *sca* (surface cell antigen) family (Roux & Raoult, 1995, 2000; Roux *et al.*, 1997; Fournier *et al.*, 1998; Sekeyova *et al.*, 2001; Ngwamidiba *et al.*, 2006), enabling the division of this genus into six groups represented by the species: *Rickettsia rickettsii*, *Rickettsia massiliae*, *Rickettsia helvetica*, *Rickettsia akari*, *Rickettsia prowazekii* and *Rickettsia canadensis*. Nevertheless, a robust and accurate phylogeny of this genus has not yet been accomplished and ambiguities remain in the phylogenetic position of some species. Moreover, for the last two decades, more novel *Rickettsia* species have been described, contributing to an increase in the complexity of rickettsial taxonomy.

As recommended by Stackebrandt *et al.* (2002), the use of several genes scattered across the bacterial genome boosts the informative level of phylogenetic data, improving the discriminatory power and the robustness of the phylogenetic relationships. Ideally, one should use data from housekeeping genes, which are ubiquitous within the population and tend to evolve at a moderate rate, and from hypervariable genes which might encode outer-surface proteins and typically evolve at an accelerated rate (Cooper & Feil, 2004). Hence, we have analysed eight target nucleotide sequences, *atpA*, *recA*, *virB4*, *dnaA*, *dnaK*, *rrl-rrf* internal transcribed spacer (ITS), *ompA* and *gltA*, selected as representative of different genomic functions believed to be active in most rickettsial species. In the present study, we compared the phylogenetic trees derived from different datasets and analysed for the first time the discriminatory capacity of concatenated sequences to infer stable phylogenetic relationships within rickettsial species. With this

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Abbreviations: dN/dS, non-synonymous/synonymous substitutions distances ratio; ITS, internal transcribed spacer; ML, maximum-likelihood; MLST, multilocus sequence typing; MP, maximum-parsimony; NJ, neighbour-joining; SFG, spotted fever group; TG, typhus group.

A table of GenBank/EMBL/DBJ accession numbers for the sequences reported in this paper is available as supplementary data with the online version of this paper.

multigenic approach we have been able to achieve a good overall phylogenetic resolution at different levels of genetic resemblance.

METHODS

Bacterial strains, culture conditions and DNA extraction.

Strains used are listed in Table 1. The rickettsiae were propagated on Vero cell (E-6 clone) monolayers in Eagle's minimal medium (MEM; Gibco-BRL) supplemented with 1% (v/v) glutamine and 8% (v/v) fetal bovine serum at 32 °C for 5–6 days. Infected cell

cultures were monitored for the presence of rickettsiae. Harvesting was done when the degree of infection was optimal, as determined by Gimenez staining (Gimenez, 1964). The cultures were frozen and unfrozen three times, and then centrifuged at 121 g for 30 min. The supernatants were recovered, then the rickettsiae were harvested by 30 min centrifugation at 7740 g and resuspended in ultra-pure water. DNA was extracted using Qiagen columns according to the DNAeasy protocol for the Animal Tissue Kit.

PCR amplification and gene sequencing. Primers used to amplify and sequence the several gene targets are listed in Table 2. PCR was performed in a 50 µl reaction mixture containing 1 pmol each primer, 200 µM each dATP, dGTP, dCTP and dTTP (Invitrogen), 1.75 U *Taq* polymerase (Invitrogen), 2 mM MgCl₂, 0.5 × BSA, 1 × *Taq* buffer and 50–100 ng genomic DNA. Amplification was carried out in a DNA thermocycler (TGradient; Biometra) using the following conditions: 5 min of initial denaturation at 96 °C, then 35 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min. The amplification was completed by holding for 5 min at 72 °C to allow complete extension of the PCR product. When no amplification product was obtained, the annealing temperature was lowered to 50 °C and the MgCl₂ concentration was raised to 2.5 mM. PCR products were visualized by ethidium bromide staining after electrophoresis in a 1% (w/v) agarose gel and their sizes were estimated by comparison with a molecular mass standard (1 kb plus DNA ladder; Gibco-BRL). The PCR products were purified using the Jet Quick-PCR Purification Kit (Genomed) as described by the manufacturer. The purified PCR products were sequenced in both directions using an automated DNA capillary sequencer CEQ 2000-XL (Beckman Coulter) and a dye-labelled dideoxy termination method (DTCS, Dye Terminator Cycle Sequencer Start Kit; Beckman Coulter). Sequences were proofread, edited and merged into composite sequences using CEQ 8000 software (Beckman Coulter). Accession numbers of nucleotide sequences are listed in supplementary Table S1 (available with the online version of this paper). All sequence data were posted at the multilocus sequence typing (MLST) website, <http://rickettsia.mlst.net> (Aanensen & Spratt, 2005).

Data analysis. For phylogenetic inference, nine different alignments were created, eight corresponding to the alignment of sequences of each gene: *atpA*, *recA*, *dnaA*, *rrl-rrf* ITS, *virB4*, *gltA*, *dnaK* and *ompA* and one alignment corresponding to the concatenation of all gene sequences. All alignments were made using the CLUSTAL W program (Thompson *et al.*, 1997). For protein-encoding genes, DNA alignments were made based on the corresponding amino acid alignments by using CodonAlign 2.0 (Hall, 2004). Aligned regions with gaps were removed from the alignment before phylogenetic analysis.

Phylogenetic analysis including neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) trees of DNA sequence alignments were conducted using PAUP* 4.0b10 software (Swofford, 2000). Genetic distances used in NJ trees are Kimura two-parameter model distances with a transition : transversion ratio of 2 : 1. Non-synonymous/synonymous substitutions distances (dN/dS) ratios, as described by Nei & Gojobori (1986), were calculated using MEGA3 software (Kumar *et al.*, 2004). Analysis of protein sequences were done using several programs present in the PHYLIP software package, version 3.6 (Felsenstein, 1989). Bootstrap analysis was made with 1000 replicates except in ML where only 100 replicates were generated. For the Bayesian approach, MrBayes software version 3.0b4 (Huelsenbeck & Ronquist, 2001) was used. Each analysis consisted of 2.0×10^7 generations from a random starting tree and four Markov chains (with default heating values) sampled every 100 generations. The first 8000 sampled trees were discarded resulting in a set of 12 000 analysed trees sampled after stationarity. To prevent reaching only apparent

Table 1. Rickettsial strains used in this study

Species	Strain	Source/geographic origin
<i>Rickettsia conorii</i>	Malish 7*	Human/South Africa
<i>Rickettsia conorii</i>	PoTiR12	Vector/Portugal
<i>Rickettsia conorii</i>	PoHuR1258	Human/Portugal
<i>Rickettsia conorii</i>	PoHuR4776	Human/Portugal
<i>Israeli tick typhus</i>	PoHuR4726	Human/Portugal
<i>Israeli tick typhus</i>	PoHuR8122	Human/Portugal
<i>Israeli tick typhus</i>	PoHuR1450	Human/Portugal
<i>Israeli tick typhus</i>	PoHuR8756	Human/Portugal
<i>Israeli tick typhus</i>	PoTiRB28	Vector/Portugal
<i>Rickettsia sibirica</i> subsp. <i>mongolotimonae</i>	PoHuR10991	Human/Portugal
<i>Rickettsia honei</i>	TT-118†	Vector/Thailand
<i>Rickettsia slovaca</i>	PoTiR24	Vector/Portugal
<i>Rickettsia slovaca</i>	PoTiR30	Vector/Portugal
<i>Rickettsia slovaca</i>	13-B†	Vector/Slovakia
<i>Rickettsia massiliae</i>	MTU1†	Vector/France
<i>Rickettsia</i> sp. Bar29	Bar29†	Vector/Spain
<i>Rickettsia aeschlimannii</i>	MC16†	Vector/Morocco
<i>Rickettsia montanensis</i>	M/56†	Vector/USA
<i>Rickettsia helvetica</i>	C9P9†	Vector/Switzerland
<i>Rickettsia helvetica</i>	PoTiR43	Vector/Portugal
<i>Rickettsia sibirica</i>	246*	Vector/Siberia
<i>Rickettsia rhipicephali</i>	3-7-6†	Vector/USA
<i>Rickettsia akari</i>	MK†	Human/USA
<i>Rickettsia bellii</i>	369L42-1†	Vector/USA
<i>Rickettsia canadensis</i>	#2678†	Vector/Canada
<i>Rickettsia africae</i>	EFS-5†	Vector/USA
<i>Rickettsia japonica</i>	YM†	Vector/Japan
<i>Rickettsia</i> sp. A-167	A-167†	Vector/Russia
<i>Rickettsia australis</i>	Philips†	Human/Australia
<i>Rickettsia</i> sp. BJ-90	BJ-90†	Vector/China
<i>Rickettsia prowazekii</i>	Madrid E*	Human/Spain
<i>Rickettsia typhi</i>	Wilmington†	Human/USA
<i>Rickettsia rickettsii</i>	R Bitterroot†	Vector/USA
<i>Rickettsia felis</i>	URRWFXCal†	Vector/France

*Genome sequence retrieved from GenBank database: AE006914, *R. conorii*; AABW00000000, *R. sibirica*; AJ235269, *R. prowazekii*.

†Strains generously provided by D. Raoult (Unité des Rickettsies, Faculté de Médecine, Université de la Méditerranée, Marseille, France). All Portuguese isolates belong to the CEVDI collection.

Table 2. Primers used for PCR amplification and sequencing

Gene	Description	Primer sequence (5'–3')*	Amplicon size (nt)	No. of nucleotides in phylogenies	dN/dS ratio†	SFG mean distance	Reference
<i>atpA</i>	ATP synthase F1 α subunit	Fw: AGTACAGACATATCGAGATGA Rv: CGACTTACCGAAATACCGAC	745	600	0.050	0.022	This study
<i>recA</i>	RecA recombination protein	Fw: CTCACCTGGGCTAGATATAGC Rv: CTTCTTATCTTTAATGGAGCC	578	482	0.039	0.027	This study
<i>virB4</i>	Virulence protein B4	Fw: TCTATAGTACATGATTCTGCT Rv: TGATTACCGAGTGTAGTATTATG	840	717	0.408	0.018	This study
<i>dnaK</i>	Heat-shock protein 70	Fw: GCATTCTAGTCATACCGCC Rv: CAAAAAATGAAAGAACTGCTGA	650	515	0.097	0.024	This study
<i>dnaA</i>	DnaA chromosomal replication initiation protein	Fw: CTTTACAATCATTACGGTG Rv: GCAACTAAGCCCCATCC	788	597	0.111	0.021	This study
<i>rrl-rrf</i> ITS	ITS	Fw: GATAGGTGGGTGTGGAAG Rv: TCGGGATGGGATCGTGTG	350	287–323	NA	0.057	Vitorino <i>et al.</i> (2003)
<i>ompA</i>	Outer-membrane protein	Fw: TGGCGAATATTCTCCAAAA Rv: AGTGCAGCATTGCTCCCCCT	532	450	1.905	0.057	Regnery <i>et al.</i> (1991)
<i>gltA</i>	Citrate synthase protein	Fw: GGGGGCCTGCTCACGGGCGG Rv: ATTGCAAAAAGTACAGTGAAC	382	380	0.112	0.023	Roux <i>et al.</i> (1997)

*Fw, Forward primer; Rv, reverse primer.

†NA, Not applicable.

stationarity, two separate runs were made for each analysis. Hierarchical likelihood ratio tests were conducted using a batch file supplied with MODELTEST 3.06 (Posada & Crandall, 1998) to provide the evolutionary models used in ML and Bayesian analysis. Based on the work by Stothard *et al.* (1994), *R. bellii* was used as outgroup.

RESULTS AND DISCUSSION

Phylogenetic relationships inferred from throughout the genome are viewed as accurate estimates of those that would be obtained from an analysis of the entire genome, since the effects of location-dependent processes in sequence evolution can be reduced by sampling sites from different regions (Cummings *et al.*, 1995). Hence, several target nucleotide sequences were selected as representative of different genomic functions (Table 2) believed to have an important role in the rickettsial life cycle, and with regard to their scattered distribution in the rickettsial chromosome (Andersson *et al.*, 1998; Ogata *et al.*, 2001). With this approach we expected to use sequences that were not contiguous in the rickettsial genome to minimize the effect of location-dependent nucleotide evolution (Cummings *et al.*, 1995). Moreover, we used both housekeeping and hypervariable genes to provide a framework for studying closely related strains as well as different species (Cooper & Feil, 2004). Although our main goal was not rickettsial strain characterization, considering that this data can be used in an MLST scheme, all sequence data were posted to an MLST

database as a focal point for future storage and retrieval of rickettsial sequence, strain and host data.

Thirty-four rickettsial strains were analysed. For *R. bellii* and *R. canadensis* the PCR amplification of some of the genes was difficult or even impossible (supplementary Table S1, available with the online version of this paper). This was not unexpected, since a greater genomic divergence is recognized for these two species, preventing the amplification of some genes even when carried out with consensus primers (designed from known *Rickettsia* spp. sequences), as described by Sekeyova *et al.* (2001).

Due to the genetic closeness of rickettsial species, nucleotide sequences were used for phylogenetic analysis instead of protein sequences. Phylogenetic trees based on amino acid sequences were drawn at a preliminary stage (results not shown), but their lack of resolution was obvious even for the most divergent gene (*ompA*).

Given that the aim of this study was to conduct a wide-ranging clarification of the phylogenetic relationships within the genus *Rickettsia* by the use of different genes, we decided to make a joint analysis based on the assembly of all the sequences, to guarantee improvement of phylogenetic tree robustness. Incongruence length difference tests have been used for data concatenation; these tests can be good indicators of different evolutionary histories and are used to

prevent the combination of incongruent data (Cunningham, 1997; Bull *et al.*, 2003). To assess the adequacy of the all-gene tree to explain the evolution of each locus, we also performed Shimodaira–Hasegawa tests with re-sampling estimated log likelihood optimization (Goldman *et al.*, 2000). In this test, different tree topologies were compared and their adequacy was tested based upon the comparison of their log likelihood values. Usually a P value ≤ 0.05 is used for the rejection of a given tree topology representing a hypothesis that might explain the data. Beyond the topology given by the all-data tree, the set of tested trees included the ML tree of each locus. Shimodaira–Hasegawa tests indicated that the tree constructed from concatenated data provides a plausible explanation for the evolutionary history of every gene (data not shown). Furthermore, single-gene trees were also obtained, enabling comparison of both approaches.

The analysis of the concatenated gene set (Fig. 1) was made using a Bayesian approach where cluster confidence is given by posterior probabilities (Huelsenbeck *et al.*, 2001).

To account for different gene-specific evolutionary rates and rate patterns, the data were partitioned for the several genes, and an appropriate evolutionary model was used for each

gene (Castoe *et al.*, 2004). In addition, the confidence of the groups obtained by this approach was also evaluated by bootstrap values taken from ML trees. However, in the latter case no partition of the data was made and a single model of DNA evolution was computed for the entire set. While the chosen model for the ITS region and *dnaK* was HKY (Hasegawa *et al.*, 1985) with gamma variation, for all the others the GTR model (Lanave *et al.*, 1984), also with gamma variation, was selected. In *atpA*, a proportion of invariable sites were considered. In the ML analysis, the GTR + G + I model was used for the entire set.

The analysis of the all-gene phylogenetic tree (Fig. 1) allows the recognition of several rickettsial groups whose phylogenetic clustering seems to be robust, with ML bootstrap values above 70 % and posterior probability values close to 1.00. Most of these groups were also recovered in MP and NJ trees (results not shown), indicating that the increase in tree resolution can be attributed to an increase in phylogenetic signal and not to the reconstruction method used. Moreover, these results are compatible with single-gene phylogenies (Fig. 2), since there are no mutually exclusive groups with significant bootstrap support. In Fig. 2, NJ trees of single-gene phylogenies are shown. Bootstrap proportions are given only for the NJ method, but

Fig. 1. MrBayes tree using concatenated sequences: *atpA*, *recA*, *dnaA*, *rrl-rrf* ITS, *virB4*, *gltA*, *dnaK* and *ompA*. The radial tree layout is shown to give a better visualization of the overall *Rickettsiae* phylogeny, and the phylogram is shown to highlight the lineage diversity within the SFG (a, in grey). In the phylogram posterior probabilities and ML bootstrap values are indicated to provide branch support. Bar, 10 % sequence divergence.

Fig. 2. For legend see opposite.

Fig. 2. NJ tree obtained from (a) *atpA*, (b) *recA*, (c) *dnaA*, (d) *rrl-rrf* ITS, (e) *virB4*, (f) *gltA*, (g) *dnaK* and (h) *ompA* sequences, and Kimura two-parameter model distances. Bootstrap proportions are given only for the NJ method. Only bootstrap values > 70% are shown. Branches leading to groups also supported by MP and ML trees are highlighted.

branches leading to groups also supported by MP and ML trees, with bootstrap values > 70 %, are highlighted.

The phylogeny obtained with all the data also has the advantage of containing information from genes with different rates of evolution that are informative at different levels. This is particularly important since the phylogenetic inferences to be made are aimed both at the comparison of genetically close taxa of the traditional SFG and of the *Rickettsia* genus as a whole. *ompA* and the ITS region, which in the SFG have a mean genetic distance (Kimura two-parameter distance with a transition:transversion ratio 2:1) of 0.057 nucleotide substitutions per site, are suitable for the clarification of phylogenetic relationships within the SFG. In contrast, phylogenies obtained from *atpA*, *recA*, *dnaA*, *virB*, *gltA* and *dnaK*, in which the mean intra-SFG group distances vary between 0.018 and 0.027, are more suited to the establishment of more basal relationships, providing good delineation of the main groups of the *Rickettsiae*. As an example, one can see that phylogenies obtained from *recA*, *virB* and *dnaK* (Fig. 2) give strong support to most of the groups suggested by Sekeyova *et al.* (2001) and reviewed by Parola *et al.* (2005).

It is generally recognized that the phylogenetic relationships of the *Rickettsiae* are characterized by the presence of several groups with one or a few identifiable individuals, and that one group, the SFG, contains most of the recognized species. It is interesting to see that all of the groups recognized so far (Sekeyova *et al.*, 2001) join each other in the middle of the phylogenetic tree, being connected by relatively short internal branches. Not surprisingly, the confidence of the clustering of any of these groups is hampered by low support in either bootstrap or posterior probability values. It should also be noted that these results do not support a monophyletic nature for the SFG. Both concatenated and single-gene sequence data indicate instead that SFG is divided into two groups: a monophyletic cluster, which includes the *R. rickettsii* group, the *R. massiliae* group (as defined by Sekeyova *et al.*, 2001) and *Rickettsia montana*, and a second group, consisting of *Rickettsia australis*, *Rickettsia akari* and *Rickettsia felis*. The only exception to this evolutionary history is presented by the *virB4* phylogeny, in which the SFG is seen as monophyletic, but without support. Regarding the *R. akari* group, only *R. australis* and *R. akari* form a well supported monophyletic cluster. This is to be expected, taking into consideration the hypothetical early separation of *R. felis* (as suggested in *virB4*, ITS and *gltA* phylogenies; Fig. 2d, e and g). As can be clearly seen from the radial tree in Fig. 1, *R. felis* branches from a central position in *Rickettsia* phylogeny. This could explain the variable phylogenetic positions or support values for *R. felis* in our study and also in other studies using different genes (Fournier *et al.*, 2003; Zhu *et al.*, 2005; Ngwamidiba *et al.*, 2006). This relatedness between both the SFG and TG rickettsiae could account for the presence in the *R. felis* genome of genes exclusively shared with *R. typhi*; this may be the reason for their

serological cross-reactivity, as pointed out by Ogata *et al.* (2005).

The previously mentioned monophyletic subgroup of the SFG rickettsiae is distinguished by the presence of many genetically close isolates. An overall good phylogenetic resolution is nevertheless possible, with many increasingly specific subgroups being recognized. In the *R. rickettsii* group, as mentioned by Sekeyova *et al.* (2001) the *R. conorii* complex is well established and can be readily subdivided into a group containing *R. conorii* Malish-like isolates, a group of Israeli-tick-typhus-like isolates and *Rickettsia* sp. A-167, the latter two being most closely related. This is in agreement with the resolution of this group as presented by Zhu *et al.* (2005). Although in this study, only Portuguese Israeli tick typhus isolates were used, *ompA* phylogenies including Israeli tick typhus strain ISTT (accession no. U83441) resulted in the same clear separation within the *R. conorii* complex (results not shown).

Another well defined monophyletic group includes not only *Rickettsia sibirica* strains, but also *Rickettsia africae*. Although the separation of these two can be made based on the all-gene phylogeny (most probably as a result of the information present in the *ompA* gene; see Fig. 2f), the proximity of *R. africae* to the *R. sibirica* group is also clear and becomes almost indistinguishable in *recA* phylogeny (Fig. 2b). The monophyly of this clade has already been recognized and it also contains *Rickettsia parkeri* and *Rickettsia* sp. strain S (Roux *et al.*, 1997; Fournier *et al.*, 1998; Roux & Raoult, 2000; Sekeyova *et al.*, 2001), which were not included in this study. Since evidence points towards it being a sister taxon to *R. conorii*, the attribution of a taxonomically defined rank such as species should be subject to further discussion.

In the remainder of the *R. rickettsii* group there is a distinct cluster with several *Rickettsia slovacae* strains (including two Portuguese isolates) and, of special notice, is the consistent position of *Rickettsia japonica* as the most divergent line in the group.

As found in the analysis of Sekeyova *et al.* (2001), the *R. massiliae* group, consisting of *R. massiliae*, *Rickettsia* sp. Bar29, *Rickettsia rhipicephali* and *Rickettsia aeschlimannii* is well supported, although only in the *ompA* single-gene tree (Fig. 2f) is it recovered with high bootstrap values by all methods. As shown in Fig. 1 the phylogenetic relationship between the *R. massiliae* group, the *R. rickettsii* group and *Rickettsia montanensis* is uncertain in this analysis. This fact, which can be seen as a difficulty in the resolution of the basal lines with regard to this diversification, gives credit to the previously recognized (Sekeyova *et al.*, 2001) variable position of *R. montanensis*, thus care should be taken when considering its inclusion in the *R. massiliae* group, as suggested recently (Parola *et al.*, 2005).

We should also draw attention to the phylogenetic position of *R. canadensis*, which is one of the most variable. In the

dnaA gene phylogeny *R. canadensis* is even hypothesized as a member of the SFG, with high bootstrap proportions and posterior probability values. Nevertheless, this phylogeny is unique because of the long phylogenetic line leading to *R. canadensis*, most probably a result of an unusually high genetic divergence of this species. Indeed, *R. canadensis* was initially considered as a member of the TG on the basis of antigenic similarities; however, phylogenetic analysis (Roux *et al.*, 1997; Fournier *et al.*, 1998) has confirmed that this species constitutes a separate group from the SFG and TG rickettsiae. Remarkably, in spite of its unclear phylogenetic position, this species seems to be closer to *R. helvetica* than to members of the TG in the concatenated data analysis (Fig. 1).

As in the TG group, *R. bellii* is phylogenetically distant from all the other members of the *Rickettsiae*, which is in agreement with its distinct genomic (Ogata *et al.*, 2006) and phenotypic (Philip *et al.*, 1983) characteristics. Indeed, *R. bellii* is believed to be the only species of the first divergent line of the genus *Rickettsia* (Stothard *et al.*, 1994). Despite its unquestionable use as an outgroup for other rickettsial species, it is impossible to recognize the order of divergence of the major groups due to the short length of internal branches, as already mentioned. This issue might be overemphasized by the missing data not only for *R. bellii* but also for *R. canadensis*. In fact, even if four gene sequences were obtained for these two species, only two of them would be common to both. This could seriously undermine inferences resulting from the presence of data from both species that would contribute to the clarification of the relatedness of the major rickettsial groups.

In conclusion, the combined use of the sequences of several housekeeping genes has led to the construction of a multi-gene phylogeny, which helps in clarifying the taxonomic structure of some groups of interest within the genus *Rickettsia*, particularly within the SFG. This group is not monophyletic, highlighting the limitations of serotyping in the delineation of taxonomic groups. Our study supports the distinctiveness of *R. montanensis*, which should thus be considered separate from the *R. massiliae* and *R. rickettsii* groups. In this analysis we have used several Portuguese isolates, which enabled us to take intraspecies variability into account. However, little resolution could be achieved at this level of relatedness. Obviously the multigenic approach to *Rickettsiae* phylogeny would be reinforced with the addition of more rickettsial species and new isolates that are constantly being described.

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