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Investigation of the phylogenetic relationships within the genus *Bartonella* based on comparative sequence analysis of the *rnpB* gene, 16S rDNA and 23S rDNA

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A variety of genes and analytical methods have been applied to the study of phylogenetic relationships within the genus *Bartonella*, but so far the results have been inconsistent. While previous studies analysed single protein-encoding genes, we have analysed an alignment containing the sequences of three important phylogenetic markers, RNase P RNA, 16S rRNA and 23S rRNA, merged by catenation, to determine the phylogenetic relationships within the genus *Bartonella*. The dataset described here comprises 13 different *Bartonella* strains, including the seven strains that are known to be human pathogens. A variety of algorithms have been used to construct phylogenetic trees based on the combined alignment and, for comparison purposes, each individual gene. Trees generated from the catenated alignment are more consistent (independent of algorithm) and robust (higher bootstrap support). It is suggested that a phylogenetic analysis incorporating the RNase P RNA, 16S rRNA and 23S rRNA be used to study the phylogenetic relationships within the genus *Bartonella*.

Keywords: 16S rDNA, 23S rDNA, RNase P RNA, phylogeny, genus *Bartonella*

INTRODUCTION

Members of the genus *Bartonella* have been associated with an broadening spectrum of clinical syndromes in both human and veterinary medicine (Anderson & Neuman, 1997; Breitschwerdt & Kordick, 2000), making the determination of the phylogenetic relationships between these strains and the identification of species-specific markers for clinical diagnostic testing imperative. The most contemporary tools used to identify micro-organisms and to study their natural relationships are the molecular approaches that utilize the sequence variability of certain genes, especially those that encode highly structured RNA molecules. The use of well-defined secondary structure in the identification of homologous residues (i.e. the alignment process) is one important advantage of RNA sequences over genes that encode proteins. In particular, comparative analysis of the 16S rDNA (Woese, 1987; Ludwig & Schleifer, 1999) is considered to be the 'gold standard' for phylogenetic analysis. However, among very closely related groups of organisms, such as the species of the genus *Bartonella*, phylogenetic differentiation at the species level and below (Fox *et al*., 1992; Suksawat *et al*., 2001; Houpikian & Raoult, 2001) is not always possible because of the high degree of 16S rDNA sequence conservation. This has led to difficulties in the identification of medically important *Bartonella* strains when using 16S rDNA-based molecular diagnostics. Alternative genes have been used successfully to increase the sensitivity and specificity of diagnostic methods for the detection of these *Bartonella* species. Useful alternative genes include a 17 kDa antigen gene (Sweger *et al*., 2000), *flaA* (Sander *et al*., 2000), *gltA* (Birtles & Raoult, 1996; Roux *et al*., 2000; Houpikian *et al*., 2001), *htrA* (Anderson & Neuman, 1997), *ftsZ* (Ehrenborg *et al*., 2000) and *groEL* (Marston *et al*., 1999), as well as the 16S–23S rDNA internally transcribed spacer sequences (Jensen *et al*., 2000; Birtles *et al*., 2000). However, when used to infer the phylogenetic relationships within the genus

The GenBank accession numbers for the 16S rDNA, 23S rDNA and *rnpB* sequences described in this work are listed in Table 1.

Bartonella, these sequences suggested conflicting relationships. Variations in the datasets, in terms of the strains and number of species used in the studies and analytical methods, have made it difficult to compare and evaluate the proposed phylogenetic relationships. Additionally, some earlier studies did not provide detailed information regarding the data analysis, including sequence accession numbers and alignments. Furthermore, all previous studies were based on the analysis of a single gene. The limitations of single gene analysis of *Bartonella* species have been recognized, especially for the use of 16S rRNA, *groEL* and *gltA* (Houpikian & Raoult, 2001).

To circumvent the limitations of single gene analysis, we performed a phylogenetic study using three phylogenetic markers, 16S rRNA, 23S rRNA and the RNA subunit of the endoribonuclease RNase P, to determine the phylogenetic relationship within the genus *Bartonella*. rRNAs are used universally for phylogenetic studies. Historically, 16S rRNA was chosen over 23S rRNA for phylogenetic studies because its smaller size made it more tractable for sequence analysis. As a result, several thousand 16S rRNA sequences are available, whereas the number of available 23S rDNA sequences remains rather small. However, the 23S rRNA contains more than twice the phylogenetic information contained in 16S rRNA (Woese, 1987; Ludwig & Schleifer, 1999). Because of vast improvements in molecular techniques, especially amplification methods and automated DNA sequencing, 23S rRNA sequences have become more easily accessible for phylogenetic analysis. RNase P RNA demonstrates a greater extent of sequence divergence per nucleotide position than the larger rRNAs. Therefore, it can be phylogenetically more informative in cases of very closely related organisms (Haas & Brown, 1998). These sequences can be used to study the phylogenetic relationships of organisms from all three kingdoms of life (Pitulle *et al.*, 1998; Woese *et al.*, 1990) or to differentiate very closely related medically important bacteria (Herrmann *et al.*, 2000). Of the 13 strains investigated in this study, *Bartonella bacilliformis*, *Bartonella clarridgeiae*, *Bartonella quintana*, *Bartonella henselae*, *Bartonella elizabethae*, *Bartonella grahamii*, *Bartonella vinsonii* subsp. *arupensis* and *Bartonella vinsonii* subsp. *berkhoffii* are known to be human pathogens (Houpikian & Raoult, 2001).

METHODS

DNA isolation and PCR amplification. Genomic DNA was isolated from bacterial cells (approx. 100 μg) by following the protocol of the Ultraclean Soil DNA isolation kit (MoBio Laboratories). All subsequent PCRs were performed on a Progene thermocycler (Techne). The 16S rDNA amplification for *B. bacilliformis* KC584 was performed as described previously (Pitulle *et al.*, 1999).

The 23S rDNA was amplified by PCR using 20 pmol each of primers 125F (5′-GATTTCCGAATGGGGMAACCC-3′; M = A or C) and 2650R (5′-CCATCCCGGTCCTCTCG-TACT-3′). Each 100 μl PCR contained dGTP, dATP, TTP and dCTP at 0·2 mM each, 2 mM $MgCl_2$ (Promega), 5 U *Taq* polymerase (Promega), 1 × buffer (Promega), 5 μl of the DNA extraction (approx. 200 ng DNA) and double-distilled water. All reactions started with an initial denaturation step of 2 min at 94 °C, followed by 30 amplification cycles (1 min at 92 °C, 1 min at 50 °C and 1 min at 72 °C) and a final extension step of 10 min at 72 °C.

The genes for the RNase P RNA were amplified by PCR using 20 pmol each of primers 59F*Bam*HI (5′-CGGGAT-CCGIIGAGGAAAGTCCIIGC-3′; I = inosine) and 347R*Eco*RI (5′-CGGAATTCRTAAGCCGGRTTCTGT-3′; R = A or G), 5 U *Taq* polymerase (Promega), 200 ng genomic DNA, 1·5 mM $MgCl_2$ and 0·2 mM each dNTP in 1 × reaction buffer (Promega). All PCRs started with an initial denaturation step for 2 min at 94 °C, followed by 35 amplification cycles (1 min at 92 °C, 30 s at 53 °C and 30 s at 72 °C) and a final extension step for 5 min at 72 °C.

PCR products were separated on a 1 % (w/v) agarose gel containing 0·5 μg ethidium bromide ml^{-1} and were visualized by UV transillumination and compared with the DNA size standards hyperladders I and IV (Denville Scientific). PCR fragments were purified according to the protocol of the QIAquick PCR purification kit (Qiagen).

Cloning of PCR products and sequencing of recombinant DNA. The purified PCR products were ligated into the pGEM-T vector system (Promega) followed by transformation of *Escherichia coli* JM109 high-efficiency competent cells according to the protocol outlined by Promega. Recombinants were selected by the blue/white colour of colonies. Plasmid DNA from several clones was isolated by using the QIAprep plasmid kit (Qiagen). Both strands of recombinant plasmid DNA (200–400 fmol) were sequenced by using the SequiTherm EXCELII Long Read DNA sequencing kit LC (Epicentre Technologies) using 1·5 pmol each of fluorescently labelled primers T7-800 (5′-GTAATA-CGACTCACTATAGGG-3′) and SP6-700 (5′-ATTTAGG-TGACACTATAG-3′). For sequencing of 16S rDNA, the primers 515F-800 (5′-GTGCCAGCMGCCGCGGTAA-3′) and 1391R-700 (5′-GACGGGCGGTGWGTRCA-3′; W = A or T) were used in addition to primers T7-800 and SP6-700. The 23S rDNAs were sequenced with primers 125F-700, 2650R-800, 1608F-700 (5′-CYACCTGTGWCGGTT-T-3′; Y = C or T) and 2235R-800 (5′-GGAGGCGACCG-CCCCAGTCAAACT-3′) in addition to T7-800 and SP6-700. After an initial denaturation step of 2 min at 92 °C, 30 sequencing cycles (30 s at 92 °C, 15 s at 50 °C and 15 s at 70 °C) were executed. The sequencing reactions were analysed by PAGE [5·5 % (v/v) for *rnpB*; 3·75 % (v/v) for rDNA] on a Li-Cor 4200 automated DNA sequencer. Sequences have been deposited in GenBank (Benson *et al.*, 2000); the accession numbers are listed in Table 1.

Sequence alignment and phylogenetic tree analysis. The previously available *Bartonella* and *Agrobacterium tumefaciens* ribosomal and RNase P RNA sequences (see Table 1) were respectively downloaded as aligned sets from the Ribosomal Database Project (Maidak *et al.*, 2001) and the RNase P database (Brown, 1999). The additional sequences were added to the alignment, inserting alignment gaps on the basis of differences in secondary structure. Sequences at either end that were not available for all sequences were discarded, leaving 313 positions in the RNase P RNA alignment, 1388 positions in the 16S rRNA alignment and 2424 positions in the 23S rRNA alignment. Considering only the *Bartonella* data, there were 36, 88 and 216 phylogenetically informative positions (i.e. positions that varied in sequence) in the RNase P, 16S rRNA and 23S

Table 1. Strains used for the phylogenetic analysis within the genus *Bartonella*

The GenBank accession numbers for sequences of the 16S rDNA, 23S rDNA and *rnpB* are listed. Sequence data that were determined in this study were deposited in GenBank under the accession numbers indicated by asterisks.

Strain	16S rDNA	23S rDNA	*rnpB*
B. vinsonii subsp. *arupensis* ATCC 700727^T	AF214558	AF410937*	AF441295*
B. clarridgeiae NCSU 94-F40^T (= ATCC 700095^T)	U64691	AF410938*	AY033649*
B. doshiae R18^T (= ATCC 700133^T)	Z31351	AF410939*	AF441294*
B. elizabethae ATCC 49927^T	L01260	AF410940*	AY033770*
B. vinsonii subsp. *berkhoffii* 93CO-1^T (= ATCC 51672^T)	L35052	AF410941*	AF375873*
B. grahamii V2^T (= NCTC 12860^T = ATCC 700132^T)	Z31349	AF410942*	AF441293*
B. henselae Houston-1^T (= ATCC 49882^T)	M73229	AF410943*	AY033897*
Bartonella strain N40	AF204274	AF410944*	AF441292*
Bartonella strain deer 159/660/1	AF373845*	AF410945*	AF376051*
B. quintana Fuller^T (= ATCC VR-358^T)	M11927	AF410946*	AY033948*
'*B. weissii*' 99-BO1	AF291746	AF410947*	AF376050*
B. vinsonii subsp. *vinsonii* Baker^T (= ATCC VR-152^T)	Z31352	AF411589*	AY033502*
B. bacilliformis KC584 (Minnick *et al.*, 1995)	AF442955*	L39095	AF440224*

rRNA alignments, respectively, or 41, 106 and 229 for those algorithms that include comparison of bases with gaps. Although the number of informative positions in the RNase P sequence alignment is relatively low, there is more variation within these positions than in the informative rRNA sequences, i.e. the information content per position is higher. The separate sequence alignments were used independently or as a merged set in which the sequence data from each strain were catenated. These alignments were used to build phylogenetic trees, using PHYLIP version 3.573c (Felsenstein, 1989) programs DNAPARS (parsimony analysis), DNAML (maximum likelihood), DNAPENNY (exhaustive parsimony searches), NEIGHBOR (neighbour-joining) and FITCH (least-squares distance matrix). In all cases, except DNAPENNY (because of the prohibitive computational demand), a consensus tree from 100 bootstrapped datasets (generated using BOOT) was generated along with the single best tree for each alignment. Global rearrangements were allowed in the construction of all trees in which this is a possibility. All trees were explicitly treated as rooted, with the *A. tumefaciens* sequences as the outgroup. In bootstrap analyses, sequences were added in random order. These alignments and the resulting output files can be accessed online at http://www.mbio.ncsu.edu/Bartonella/.

RESULTS AND DISCUSSION

The phylogenetic analysis of certain DNA sequences is a powerful tool for the determination of the natural relationships between micro-organisms. Although the quality of phylogenetic trees can be estimated by bootstrap analysis, these values alone do not entirely reflect the correctness of the trees. The analysis also depends on the type of gene used, its molecular clock tempo and mode and, most importantly, a meaningful sequence alignment. In the case of RNA sequences, alignments are based on homology identified by comparative analysis (Gutell, 1993; James *et al.*, 1989) rather than simply maximizing sequence similarity, as is customary in protein alignments.

We have isolated and determined the nucleotide sequences of the genes for 13 RNase P RNAs, 12 23S rRNAs and two 16S rRNAs from 13 *Bartonella* strains (Table 1). The PCR products for the RNase P RNAs represent about 90 % of the complete RNA molecules, all of which resemble *E. coli*-like RNase P RNAs (A-type) with minimal differences in size, ranging from 345 to 349 nt. The RNase P RNAs, at $\geqslant$ 94 % sequence identity, are the least conserved of the sequences, and the differences are dispersed in the RNA sequence. Therefore, the use of *Bartonella* RNase P RNA for diagnostic testing based on size-selective or species-specific PCR is unlikely to be readily useful. We did not find a single restriction endonuclease or any combination of any two of these enzymes that can digest all 13 partial *rnpB* genes to generate unique restriction-fragment patterns. However, because of its small size, sequencing of the entire amplified *rnpB* gene on both strands is relatively easy and is a very accurate method for clear discrimination of the 13 *Bartonella* strains investigated in this study.

Of the two rRNAs, the 23S rRNAs show sequence identities of $\geqslant$ 96 %, whereas the 16S rRNAs are $\geqslant$ 98 % identical. We determined 1406 and 1407 nt, respectively, of the 16S rDNAs derived from *Bartonella* sp. deer isolate 159/160/1 and *B. bacilliformis* KC584. The sequence and size differences (2404–2451 nt) in the 23S rDNA are more significant for diagnostic PCR testing than those seen in the 16S rDNAs or the RNase P RNAs. The use of 23S rDNA for diagnostic purposes will be presented in a forthcoming publication.

We combined our sequences with existing 16S rDNA and 23S rDNA data extracted from GenBank (Benson *et al.*, 2000) to create a single alignment set based on the secondary structures of these molecules. Only those regions defined as homologous on the basis of

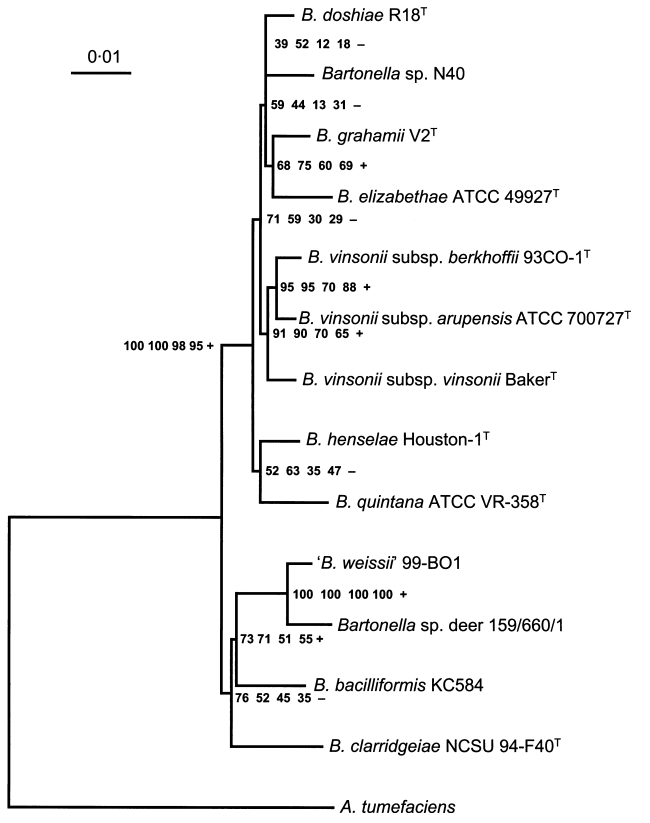

Fig. 1. Phylogenetic tree of *Bartonella* species based on the combined RNase P RNA, 16S and 23S rRNA sequence alignment. *A. tumefaciens* serves as the outgroup in this tree. The tree shown was generated using FITCH (see text), which best matches the consensus bootstrap trees. The horizontal axis is the estimated evolutionary distance. The four numbers shown at each node are the numbers of times that node appears among 100 bootstrapped trees: FITCH, NEIGHBOR, DNAPARS and DNAML from left to right. The final + or – indicates whether or not this node appeared in the single most parsimonious tree identified using DNAPENNY. Bar, estimated evolutionary distance of 0·01 substitutions per position.

conserved secondary structure were used for the analysis. After the combination of all three genes into one alignment, we had 340 (or 376, for some algorithms) phylogenetically informative nucleotide positions for each strain. We restricted the results presented to those phylogenetic trees only that were derived from the combined datasets (Fig. 1). We initially included the 16S–23S internally transcribed spacer sequences from the 13 *Bartonella* strains in our analysis, but we were unable to produce a phylogenetically meaningful alignment because of the large extent of sequence divergence.

The deepest division among *Bartonella* species, e.g. the root, is probably between the group defined by '*Bartonella weissii*', *Bartonella* sp. deer isolate 159/660/1, *B. bacilliformis* and perhaps *B. clarridgeiae* and the rest of the organisms (Fig. 1). '*B. weissii*', *Bartonella* sp. deer isolate 159/660/1, *B. bacilliformis* and probably *B. clarridgeiae* form a distinct monophyletic subgroup, with '*B. weissii*' and the *Bartonella* sp. deer isolate 159/660/1 as the closest relatives in this group, which is highly supported in bootstrap analyses of the combined data as well as by the 16S and 23S rDNA sequences independently. The phylogenetic analysis based on RNase P RNA sequences also supports the presence of this group but suggests that *Bartonella* sp. deer isolate 159/660/1 and *B. clarridgeiae* are the closest relatives. The placement of *B. clarridgeiae* is less consistent than that of the other sequences; bootstrapped trees were nearly evenly split, regardless of the algorithm used, between placement along with '*B. weissii*', *Bartonella* sp. deer isolate 159/660/1 and *B. bacilliformis* or placement alone as the deepest branch in the *Bartonella* tree. These alternatives represent a difference in the placement of the outgroup branch, not a difference in the branching arrangement amongst the *Bartonella* species. The alternative placements of *B. clarridgeiae* are both well represented in bootstrap analyses from the 16S, 23S and the combined data. However, *B. bacilliformis* and *B. clarridgeiae* are the only known *Bartonella* species that have flagella (Sander *et al.*, 2000). Recently, '*B. weissii*', isolated from domestic cats, has also been reported as a flagellated strain (Regnery *et al.*, 2000). Although it is not always possible to correlate phenotypic features with phylogenetic relationships based on genotypes, the presence of flagella may be a general feature of this group.

Previous studies have grouped *B. vinsonii* subsp. *vinsonii*, *B. vinsonii* subsp. *berkhoffii* (Kordick *et al.*, 1996) and *B. vinsonii* subsp. *arupensis* (Welch *et al.*, 1999) as subspecies of *B. vinsonii* (Sweger *et al.*, 2000; Roux *et al.*, 2000; Houpikian *et al.*, 2001). However, the data differed with regard to the relationship of this species to other strains of the genus *Bartonella*; the resulting trees were generally not well supported by bootstrap values, and only one study included all three subspecies (Houpikian *et al.*, 2001). On the basis of our analysis, the three subspecies to *B. vinsonii* form a monophyletic cluster. The 23S rDNA-derived data and the combined data clearly place *B. vinsonii* subsp. *arupensis* and *B. vinsonii* subsp. *berkhoffii* as close relatives, which is well supported by high bootstrap values. *B. vinsonii* subsp. *vinsonii* branches deeper within this group, and this placement is also well supported. The RNase P RNA analysis groups together *B. vinsonii* subsp. *arupensis* and *B. vinsonii* subsp. *vinsonii* but, depending on the algorithm used, *B. vinsonii* subsp. *berkhoffii* can occupy different places within the trees, either within or outside this group, reflecting uncertainty in some of the deeper, short branches in this part of the trees. The 16S rDNA analysis groups *B. vinsonii* subsp. *arupensis* and *B. vinsonii* subsp. *berkhoffii* together as well, but *B. vinsonii* subsp. *vinsonii* is not as definitively grouped with them.

B. elizabethae and *B. grahamii*, both of which were isolated from rodents, form a subgroup that is best supported by the combined analysis and by the RNase

P RNA data. The 16S rDNA data suggest this relationship as well, although the bootstrap support is low. The relationship of *Bartonella* strain N40 (Bermond *et al*., 2000) and *Bartonella doshiae*, which are also rodent isolates, is not as clear; neither organism can be unequivocally assigned to any particular place in the tree. Although most trees place both organisms into one subgroup, this relationship is not well supported by bootstrap analysis. If these organisms are specifically related, the relationship is an indistinct one. *Bartonella* strains N40, IBS 325^T and IBS 358 are three bacterial strains that were isolated from *Apodemus* spp. Their partial 16S rDNA sequences as well as their partial *gltA* gene sequences were found to be identical. On the basis of additional biochemical and molecular tests, all three strains were considered to represent the same *Bartonella* species, *Bartonella birtlesii*, although IBS 325^T became the type strain (Bermond *et al*., 2000).

B. quintana and *B. henselae* seem to represent independent branches within the tree, although they appear together in a subset of trees. The relative branching order of these sequences is not well supported by bootstrap values. Determining the relationship between these organisms and the remaining *Bartonella* species will require sequence data from additional isolates of this genus.

The use of 16S rDNA data analysis remains the generally accepted gold standard when studying phylogenetic relationships. The high degree of 16S rDNA sequence identity within the genus *Bartonella*, however, has been recognized as a problem because branching arrangements of the trees often elicit very little statistical support. It is doubtful whether phylogenetic relationships should be based solely on 16S rRNA in cases where sequence identities are $\geqslant 99\,\%$ (Drancourt *et al*., 2000). Although several protein-encoding genes have been suggested as models for the study of phylogenetic relationships within the genus *Bartonella*, the citrate synthase gene (*gltA*) has been most widely used as an alternative to 16S rDNA analysis. However, the use of *gltA* consistently resulted in low bootstrap support for the resulting trees. Although *gltA* has been used successfully to differentiate *Bartonella* species for diagnostic purposes, the phylogenetic interpretation of these diagnostically important changes in the DNA sequences remains unclear.

Although single gene analysis has been used extensively to study the phylogenetic relationships within the genus *Bartonella*, the present study clearly demonstrates that predictions concerning the natural relationships based on only one gene are not always possible. Results are even more confusing when two or more genes are analysed independently and relationships are concluded from comparisons of the separate trees (Houpikian & Raoult, 2001).

In contrast, the combined use of the phylogenetic markers RNase P RNA, 16S rRNA and 23S rRNA can contribute significantly to clarifying the phylogenetic relationships among *Bartonella* species. The alternative phylogenetic algorithms used resulted in more consistent tree topologies, and the statistical support for the suggested relationships is generally better than for any single gene, as indicated by higher bootstrap values. One might expect the molecules with the lowest or highest sequence similarities to skew the results. However, there was no single gene in our study that consistently dominated the data analysis. Also, because of its size, the combined dataset is not as susceptible to sequencing errors and allelic heterogeneity as data derived from a single gene.

In conclusion, we suggest that a combined phylogenetic analysis incorporating the RNase P RNA, 16S rRNA and 23S rRNA be used to study the phylogenetic relationships within the genus *Bartonella*. Because of their phylogenetic value, all three genes could be used when describing a novel *Bartonella* species or when novel *Bartonella* isolates are reported.

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