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Phylogenetic classification of *Bartonella* species by comparing *groEL* sequences

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***Bartonella* is a bacterial genus classified in the *α-Proteobacteria* on the basis of 16S rDNA sequence comparison. The highly conserved heat-shock chaperonin protein, GroEL, has proved to be a valuable resolving tool to classify ten *Bartonella* species. The *groEL* gene was amplified and sequenced from ten *Bartonella* isolates: *Bartonella alsatica, Bartonella vinsonii* subsp. *arupensis, Bartonella taylorii, Bartonella tribocorum, Bartonella birtlesii, Bartonella henselae* Marseille (URLLY8), *B. henselae* (90-615), *B. henselae* (Fizz), *B. henselae* (CAL-1) and *B. henselae* (SA-2). Then, phylogenetic relationships were inferred between our isolates and eight other species and subspecies from the comparison of both 16S rDNA and *groEL* sequences using parsimony, neighbour-joining and maximum-likelihood methods. By using *groEL* sequences, the first reliable classification of most known *Bartonella* species and subspecies was established. Four strongly supported subgroups were distinguished: firstly, the two human pathogens *B. henselae* and *Bartonella quintana*; secondly, a cluster including four rodent isolates, *Bartonella elizabethae, B. tribocorum, Bartonella grahamii* and *B. taylorii*; thirdly, a cluster including the *B. vinsonii* subspecies (*B. vinsonii* subsp. *vinsonii, arupensis* and *berkhoffii*); and lastly, *B. birtlesii* and '*Bartonella weissi*'. '*Bartonella washoensis*', *B. alsatica, Bartonella doshiae, Bartonella bacilliformis* and *Bartonella clarridgeiae* did not reliably cluster with any other *Bartonella* species. In addition, the *groEL* gene was shown to be useful in subtyping six *B. henselae* isolates into three variants: Houston, Marseille and Fizz.**

Keywords: *Bartonella*, 16S rDNA, *groEL*, phylogeny, subtyping

INTRODUCTION

Bacteria within the genus *Bartonella* are aerobic, Gram-negative, fastidious, oxidase-negative, slow-growing, pleiomorphic organisms, which belong to the *α-Proteobacteria* on the basis of their 16S rDNA sequences (Brenner *et al*., 1993; Birtles *et al*., 1995). These bacteria are considered to be emerging pathogens (Anderson & Neuman, 1997). Currently, 18 *Bartonella* species are recognized. All of them are associated with mammalian hosts. *Bartonella taylorii*, *Bartonella elizabethae*, *Bartonella tribocorum* and *Bartonella birtlesii* were isolated from rats (Birtles *et al*., 1995; Brenner *et al*., 1993; Heller *et al*., 1998; Bermond *et al*., 2000); *Bartonella grahamii*, *Bartonella vinsonii* subsp. *vinsonii* and *Bartonella doshiae* were recovered from voles (Birtles *et al*., 1995; Brenner *et al*., 1993); *B. vinsonii* subsp. *arupensis* was isolated from mice (Welch *et al*., 1999); *Bartonella alsatica* was isolated from rabbits (Heller *et al*., 1999); '*Bartonella weissi*', *Bartonella clarridgeiae*, *Bartonella henselae* and *Bartonella koehlerae* were obtained from cats (Droz *et al*., 1999; Kelly *et al*., 1998; Koehler *et al*., 1994; Lawson & Collins, 1996); *B. vinsonii* subsp. *berkhoffii* was cultivated from dogs (Breitschwerdt *et al*., 1995) and from coyotes (Chang *et al*., 2000); '*Bartonella washoensis*' was evidenced in rodents (R. L. Regnery, personal communication); and *Bartonella quintana* and *Bartonella bacilliformis* were isolated from humans (McNee *et al*., 1916; Gray *et al*., 1990). As *Bartonella* species express few remarkable phenotypic characteris-

Abbreviation: ITS, intergenic spacer.

tics, their precise identification and phylogenetic classification has mainly relied on the study of various genes. The 16S rDNA sequence, which was considered to be one of the most useful and informative tools for the identification and phylogenetic studies of bacteria (Olsen & Woese, 1993), was the first gene to be studied but has failed to establish a reliable phylogeny of *Bartonella* species. The high degree of conservation of this gene led to a small number of informative sites in its sequence. Thus, it does not seem to be a good tool to reveal a precise and statistically supported phylogeny at the species level (Fox *et al*., 1992; Hasegawa & Hashimoto, 1993; Teichmann & Mitchison, 1999). Other genes have been investigated to classify *Bartonella* species. The 16S–23S rDNA intergenic spacer (ITS) region (Roux & Raoult, 1995) and the citrate synthase-encoding gene (*gltA*) (Birtles & Raoult, 1996) provided better bootstrap values for the nodes than those obtained with the 16S rDNA sequence. Currently, the most recent and reliable classification of *Bartonella* species was established by Marston *et al*. (1999) who, using the 60 kDa heat-shock protein-encoding gene (*groEL*), which is one of the two highly conserved components of the heat-shock chaperonin response system *groES* (Hsp10)/*groEL* (Hsp60) (Mayhew & Hartl, 1996), established the relationships between nine *Bartonella* strains.

In the present study, sequences of the major portion of the *groEL* gene from ten additional *Bartonella* isolates were determined. From the alignment of these sequences and those available for another eight species and subspecies, the phylogenetic relationships within the *Bartonella* genus were inferred. The utility of this gene as a tool in subtyping *B. henselae* and *B. quintana* isolates was also tested.

METHODS

***Bartonella* strains and DNA extraction.** The strains used in this study are summarized in Table 1. *Bartonella* isolates were grown on 5% sheep blood agar (bioMérieux) at 37 °C in a 5% CO_2-enriched atmosphere. Bacteria were harvested after 7 d cultivation and DNA was extracted using the Chelex method (de Lamballerie *et al*., 1992). Supernatants containing the genomic DNAs were stored at 4 °C until their use as templates in PCR.

PCR amplification and DNA sequencing. Primers used for amplification and sequencing are presented in Table 2. Primer positions are numbered relative to the *groEL* gene of *B. bacilliformis* (Table 1). Primers were selected using the Primer3 software (Rozen & Skaletsky, 2000; code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html) and were purchased from Eurobio. PCRs were carried out in PTC-200 automated thermocyclers (MJ Research) using a *Taq* DNA polymerase kit (Gibco-BRL) and primers BbHS1630.n and HSPF1D (Table 2). The 25 µl reaction mixture consisted of (final concentration): primers (0·5 pmol μl^{-1} each), $MgCl_2$ (1·6 mM μl^{-1}), dNTP (dATP, dCTP, dGTP and dTTP) (0·2 mM μl^{-1} each), 2·5 µl buffer 10 × and *Taq* DNA Polymerase enzyme (0·03 U μl^{-1}), 5 µl DNA preparation and sterile water. PCR amplification was performed under the following conditions: a 3 min denaturation at 94 °C was followed by 40 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 54 °C and extension for 90 s at 72 °C. The amplification was completed by holding for 7 min at 72 °C to allow complete extension of the PCR products. PCR products were separated by electrophoresis on 1% agarose gels, visualized by staining with ethidium bromide and then purified using the QIAquick PCR purification kit (QIAGEN) as described by the manufacturer. PCR products were sequenced in both directions using the d-Rhodamine Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) as described by the manufacturer. Each reaction was carried out using 5 µl purified DNA (~ 200 ng), d-Rhodamine mix (4 µl) and 1 µl primer (10 pmol); sequencing primers are reported in Table 2. Conditions used for sequencing were 30 cycles of denaturation for 20 s at 95 °C, annealing for 10 s at 50 °C and extension for 2 min at 60 °C. Reaction products were mixed with 80 µl 70% ethanol and 0·5 mM $MgCl_2$ and, after being held for 20 min at room temperature in the dark, precipitated DNA was collected by centrifugation for 25 min at 3000 r.p.m. at room temperature. The DNA pellet was dried and resuspended in either 3 µl 1/4 (v/v) formamide/1% bromophenol blue solution and then denatured by heating for 2 min at 95 °C or 12 µl Template Suppression reagent (Perkin Elmer). Sequencing products were resolved using an ABI 377 or an ABI 310 automated sequencer (Perkin Elmer).

Analysis of sequences and construction of phylogenetic trees. Sequence analysis was performed with the software packages ABI Prism DNA Sequencing Analysis Software version 3.0 (Perkin Elmer) and multisequence alignment was made with CLUSTAL W software, version 1.81 (Thompson *et al*., 1994). Phylogenetic trees were obtained from DNA sequences by using the maximum-parsimony method (DNAPARS software in PHYLIP; Felsenstein, 1989), distance methods (DNADIST, distance matrix with Kimura two-parameter or Jukes–Cantor parameters; and NEIGHBOR, neighbour-joining) and the maximum-likelihood method (DNAMLK software in PHYLIP). Phylogenetic trees were inferred from amino acid sequences using the maximum-parsimony method (PROTPARS software in PHYLIP) and distance methods (PROTDIST, dayhoff PAM matrix or Kimura formula; and NEIGHBOR, neighbour-joining) Bootstrap replicates were performed to estimate the node reliability of the phylogenetic trees obtained by the three methods (Brown, 1994). Bootstrap values were obtained from 100 trees (Efron *et al*., 1996) generated randomly with SEQBOOT and CONSENSE in the PHYLIP software package. Only values above 90 were considered significant. Trees were drawn using the TREEVIEW version 1.5 (Page, 1996) software. *Agrobacterium tumefaciens* and *Brucella abortus*, two other α-*Proteobacteria*, were used as the outgroup in all our phylogenetic trees (Table 1). Only the neighbour-joining tree is presented in this article.

By comparing *groEL* sequences of *B. henselae* isolates (Marseille, SA-2, Fizz, CAL-1, 90-615 and Houston-1^T) and *B. quintana* isolates (Fuller and Oklahoma), the utility of this gene in subtyping these two *Bartonella* species was investigated. Sequences were aligned using the CLUSTAL W software. A phylogenetic tree was constructed from the distance matrix generated by the neighbour-joining method. *B. bacilliformis* was used as outgroup.

Table 1. Bacterial strains and sequences used in this study

Species/strain	Collection no./source*	Sequence accession no.:	
		16S rRNA	*groEL*
Agrobacterium tumefaciens (type strain)		D14500	X68263
Bartonella alsatica IBS 382^T	CIP 105477^T	AJ002139	AF299357
Bartonella bacilliformis (type strain)		Z11683	Z15160
Bartonella birtlesii IBS 325^T	CIP 106294^T	AF204274	AF355773
Bartonella clarridgeiae 94-F40^T		U64691	AF014831
Bartonella doshiae (type strain)		Z31351	AF014832
Bartonella elizabethae (type strain)		L01260	AF014834
Bartonella grahamii (type strain)		Z31349	AF014833
Bartonella henselae CAL-1	CDC		AF304020
Bartonella henselae Fizz			AF304022
Bartonella henselae Houston-1^T		M73229	AF014829
Bartonella henselae Marseille^T	CIP 104756^T	AF214556	AF304019
Bartonella henselae SA-2	CDC		AF304021
Bartonella henselae 90-615	CDC		AF304023
Bartonella koehlerae C-29^T	ATCC 700693^T	AF076237	ND
Bartonella quintana Fuller^T	ATCC VR 358^T	M11927	
Bartonella quintana Oklahoma^T	CDC		AF014830
Bartonella taylorii M6^T	NCTC 12861^T	Z31350	AF304017
Bartonella tribocorum IBS 506^T	CIP 104576^T	AJ003070	AF304018
Bartonella vinsonii subsp. *arupensis* OK 94-513^T	ATCC 700727^T	AF214558	AF304016
Bartonella vinsonii subsp. *berkhoffii* (type strain)		U26258	AF014836
Bartonella vinsonii subsp. *vinsonii* G7464^T		M73230	AF014835
'*Bartonella washoensis*' nvh1		AF070463	AF071193
'*Bartonella weissi*' FC7049UT		AF199502	AF071194
Brucella abortus (type strain)		X13695	M82975

ND, Not determined.

* Abbreviations: CIP, Collection de l'Institut Pasteur; Paris, France; CDC, Centers for Disease Control, Atlanta, GA, USA; ATCC, American Type Culture Collection, Manassas, VA, USA; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK.

Table 2. Primers used for PCR and/or sequencing

The target gene in all cases was *groEL*.

Primer*	Primer sequence	Position (direction)†
HSPF1d (ap, s)	5′-GAACTNGAAGATAAGTTNGAA-3′	180 (→)
BbHS1630.n (ap, s)‡	5′-AATCCATTCCGCCCATTC-3′	1668 (←)
HSP1 (s)	5′-GGAAAAAGTNGGCAATGAAG-3′	501 (→)
HSP2 (s)	5′-GCNGCTTCTTCACCNGCATT-3′	1412 (←)
HSPS1 (s)	5′-AAGCNCCNGGNTTTGGTGA-3′	865 (←)
HSPS2 (s)	5′-TCACCAAANCCNGGNGCTT-3′	846 (→)
HSPF2d (s)	5′-GAAAGANCGNGTNGATGAT-3′	1203 (→)
HSPR2d (s)	5′-GTNATNAGAAGNCTNGCAAT-3′	1575 (←)

* Abbreviations: ap, amplification primer; s, sequencing primer.

† Positions are numbered relative to the *groEL* gene of *Bartonella bacilliformis* (accession no. Z15160). Arrows indicate direction of primers (→, forward; ←, reverse).

‡ Marston *et al.* (1999).

RESULTS

Phylogeny of *Bartonella* species based on comparison of 16S rDNA sequences

To obtain a contiguous alignment, 12 bases (positions 154–165) of the 16S rDNA sequence of *B. vinsonii* subsp. *berkhoffii* were removed. Trees inferred using the maximum-parsimony, the neighbour-joining and maximum-likelihood methods showed similar organization. Only one cluster, formed by *B. elizabethae* and *B. tribocorum*, was supported by elevated bootstrap values (95, 93 and 90 % using the methods cited above, respectively). *B. henselae* Marseille and *B. henselae* Houston-1^T formed one reliable cluster only when using the neighbour-joining method (98 %). In general, most of the *Bartonella* clusters obtained lacked statistical support, whereas branching of the two outgroup bacteria, *Brucella abortus* and *A. tumefaciens*, was statistically supported (100 %; data not shown).

Phylogeny of *Bartonella* species based on the comparison of *groEL* DNA sequences

Contiguous multiple alignment of 1188 bp (72 % of the entire gene sequence) of the *groEL* gene was carried out. Phylogenetic analysis using the maximum-parsimony, neighbour-joining (Fig. 1) and maximum-likelihood methods provided similar and reliable organizations. The neighbour-joining-derived trees using Jukes–Cantor or Kimura two-parameter showed similar organization, but when using the second correction method, higher bootstrap values were obtained, which were used below. Four reliable clusters were characterized. A cluster including the three *B. vinsonii* subspecies (*B. vinsonii* subsp. *vinsonii*, *arupensis* and *berkhoffii*) was obtained using all three methods with significant bootstrap values (95, 93 and 90 %, respectively). However, within this cluster, the organization of the three subspecies varied. Using neighbour-joining and maximum-likelihood, *B. vinsonii* subsp. *vinsonii* clustered with *B. vinsonii* subsp. *arupensis*, whereas using maximum-parsimony, it clustered with *B. vinsonii* subsp. *berkhoffii*. This variation was reflected by bootstrap values of 47, 50 and 71 %, respectively. In a second cluster, the two *B. henselae* subspecies (Houston-1^T and Marseille) and *B. quintana* grouped together in all trees. This cluster was supported by higher bootstrap values using the neighbour-joining method (90 %) than both parsimony and maximum-likelihood (83 and 63 %, respectively). *B. elizabethae* and *B. tribocorum* grouped together using all three methods with significant bootstrap values (94, 100 and 100 %, respectively), which was also the case for *B. grahamii* and *B. taylorii* (bootstrap values of 100, 100 and 99 %, respectively). Using all three methods, these two groups formed a third statistically

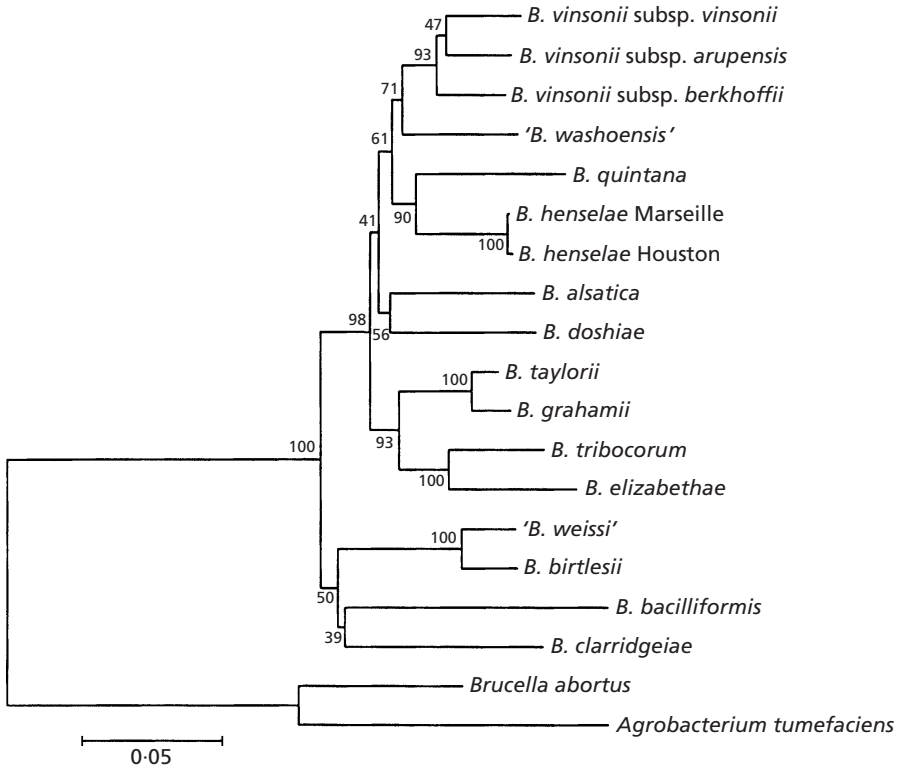

Fig. 1. Neighbour-joining tree (based on the Kimura two-parameter model of nucleotide substitution) based on the *groEL* nucleotide sequences. Bootstrap values at the tree nodes are based on 100 replicates. The tree was rooted with *Agrobacterium tumefaciens*. Bar, 5 % divergence.

supported cluster (89, 93 and 94 %, respectively). *B. birtlesii* and '*B. weissi*' formed the fourth cluster, which was statistically supported when using all three methods (100 % each). However, these bacterium clustered with *B. clarridgeiae* with low bootstrap values (53, 50 and 37 %, respectively). The grouping of *B. bacilliformis*, *B. alsatica*, '*B. washoensis*' and *B. doshiae* observed in all three methods was not reliable. As mentioned above, classification of the two species outside the genus *Bartonella*, *Brucella abortus* and *A. tumefaciens*, was reliable and consistent in all three trees (100 % each). The phylogenetic organization obtained using the various analysis methods was supported by nucleotide substitutions at informative sites observed in the sequence alignment (data not shown). When masking the third nucleotide position, the branching order was not modified (data not shown). The *groEL* gene of *B. koehlerae* could not be amplified, but additional attempts are currently being made to resolve this problem.

Phylogeny of *Bartonella* species based on comparison of the GroEL amino acid sequences

The level of amino acid sequence similarity among the studied strains varied from 90·9 % between *B. bacilliformis* and *B. taylorii* to 99·1 % between *B. vinsonii* subsp. *vinsonii* and *berkhoffii*. In the neighbour-joining-calculated trees, three clusters, *B. henselae* subsp. Marseille and Houston-1^T, *B. birtlesii* and '*B. weissi*', and *B. vinsonii* subsp. *vinsonii* and *berkhoffii*, were supported by sufficient bootstrap values [90, 100 and 89 %, respectively, when using dayhoff PAM matrix and 94, 100 and 91 %, respectively, when using the Kimura formula (data not shown)]. When using the PROTPARS method, these three clusters where established with significant bootstrap values: 72, 100 and 93 %, respectively (data not shown).

Subtyping of *B. henselae* and *B. quintana* species using *groEL* gene sequences

The *groEL* gene sequences of *B. quintana* strain Oklahoma (Marston *et al.*, 1999) and strain Fuller were 100 % identical, whereas *groEL* gene sequences from five *B. henselae* strains (Marseille, SA-2, Fizz, CAL-1 and 90-615) were determined and compared to that of *B. henselae* strain Houston-1^T. Three *groEL* variants were identified and were arranged in two clusters (data not shown). The first one was statistically supported and included *B. henselae* strains Houston-1^T, 90-615 and SA-2, whereas the second cluster did not have statistical support and it included *B. henselae* strains Marseille, CAL-1 and Fizz. Within the Marseille cluster, the sequence from strain Fizz could be differentiated from those of the two other strains.

DISCUSSION

Comparison of 16S rDNA sequences has led to many taxonomic reassessments within the genus *Bartonella* (Brenner *et al.*, 1993; Birtles *et al.*, 1995). In particular, the genera *Bartonella*, *Rochalimaea* (Brenner *et al.*, 1993) and *Grahamella* (Birtles *et al.*, 1995) were unified. However, although shown to be useful at the genus level (Olsen & Woese, 1993), the reliability of the 16S rDNA sequence as a tool for phylogenetic studies at the species level has been questioned (Fox *et al.*, 1992; Hasegawa & Hashimoto, 1993). Alternative genes that may be used for phylogenetic purposes should be both highly conserved (i.e. housekeeping genes) and sufficiently variable to allow species identification (Olsen & Woese, 1993). Several empirically chosen genes have been used in an attempt to classify the *Bartonella* species. The *gltA* (Birtles & Raoult, 1996) and the 16S–23S ITS (Roux & Raoult, 1995; Jensen *et al.*, 2000) genes have been used. Resulting trees had a better resolution than those inferred from 16S rDNA sequences, but many branches lacked a statistical support. A recent ITS-derived classification of *Bartonella* species performed in our laboratory showed reliable tree organizations, similar to *groEL*-derived trees (Houpikian & Raoult, 2001). The importance of the *groEL* gene as a sensitive and valuable tool for bacterial species phylogeny (Viale *et al.*, 1994) was recently highlighted. GroEL (Hsp60) is essential for protein folding, assembly and secretion (Mayhew & Hartl, 1996). As such, this gene is present in both prokaryotes and eukaryotes. It was used in phylogenetic analysis of *Borrelia* species (Wallich *et al.*, 1992), mitochondria of *Euglena gracilis* (Yasuhira & Simpson, 1997), *Staphylococcus* species (Kwok *et al.*, 1999), photosynthetic prokaryotes (Gupta *et al.*, 1999), *Ehrlichia* species (Sumner *et al.*, 1997; Shibata *et al.*, 2000) and *Bartonella* species (Marston *et al.*, 1999). In the latter study, the usefulness of this gene has been highlighted for the phylogenetic classification of *Bartonella* species, but many species were not used in this study. In the present study, *groEL* sequences were used to assess the classification of most of the currently known species of the genus *Bartonella* and their utility in the subtyping of *B. henselae* and *B. quintana* isolates was investigated. By comparing phylogenetic trees derived from 16S rDNA sequences, it was confirmed that this gene was unable to resolve the relationships within the genus *Bartonella* as most branches lacked statistical support and only one reliable cluster, formed by *B. elizabethae* and *B. tribocorum*, was established. In contrast, trees generated using *groEL* sequences were much more informative. All three phylogenetic analysis methods provided similar and reliable topologies. *Bartonella* species were distributed in four clusters supported by significant bootstrap values. The first cluster included the worldwide distributed human pathogens *B. henselae* Houston-1^T and Marseille and *B. quintana*. The second cluster included *B. elizabethae*, *B. tribocorum*, *B. grahamii* and *B. taylorii*. Although all are associated with rodents, these species were not geographically homogeneous, as three have been isolated in Europe and one in the USA (*B. elizabethae*). The third cluster contained the three *B. vinsonii* subspecies: *B. vinsonii* subsp. *vinsonii*, *arupensis* and *berkhoffii*, all isolated in North America. In the fourth

cluster, *B. birtlesii* and '*B. weissi*' were grouped with reliable bootstrap values when using all methods for nucleotide and amino acid sequences analysis. However, phylogenetic association of these two species did not correlate with their host or geographical origin. *B. bacilliformis*, which has a limited geographical distribution, did not reliably cluster with any other *Bartonella* species. *B. alsatica* and *B. doshiae*, which have both been isolated from rodents in Europe, clustered together using neighbour-joining methods, but their group was not statistically supported. The classification of all other *Bartonella* species studied was uncertain. In the future, the study of novel isolates may strengthen the classification of these *Bartonella*. Some of the clusters inferred from comparison of *groEL* sequences were consistent with previous studies. *B. grahamii* and *B. elizabethae*, and *B. quintana* and *B. henselae* clusters had previously been established using *gltA* sequences (Birtles & Raoult, 1996). These two clusters, as well as that formed by *B. vinsonii* subsp. *berkhoffii* and *B. vinsonii* subsp. *vinsonii*, were also described by Marston *et al.* (1999) based on *groEL* sequence analysis. When examining amino acid sequences deduced from *groEL* nucleotide sequences, a reliable phylogenetic organization for bartonellae was not obtained, although three reliable branches were established, which may be explained by the high degree of amino acid sequence conservation among *Bartonella* species. Additionally, this gene was useful in the subtyping of *B. henselae* isolates, but failed with those of *B. quintana*, confirming that this species is more homogeneous than *B. vinsonii* or *B. henselae*. In conclusion, the *groEL* gene was shown to be more reliable than any of the previously studied tools to infer precise phylogenetic relationships among *Bartonella* spp.

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