Diversity of bartonellae associated with small mammals inhabiting Free State province, South Africa

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The prevalence and diversity of bartonellae infecting the blood of 10 small mammal species inhabiting nine Nature Reserves of the Free State province, South Africa, was assessed using phenotypic, genotypic and phylogenetic methods. Of 86 small mammals sampled, 38 animals belonging to five different species yielded putative bartonellae. Thirty-two isolates were confirmed as bartonellae and were characterized by comparison of partial citrate synthase gene (*gltA*) sequences. Phylogenetic reconstructions derived from alignment of these sequences with those available for other bartonellae indicated that the South African rodent-associated isolates formed two distinct clades within the radius of the genus *Bartonella*. One of these clades also included recognized *Bartonella* species associated with rodents native to Eurasia but not to the New World, whereas the second clade contained exclusively isolates associated with South African rodents. Comparison of *gltA* sequences delineated the isolates into a number of ecologically distinct populations and provided an indication that a combination of phylogenetics and the identification of sequence clusters in housekeeping protein-encoding genes could be developed as a key criterion in the classification of bartonellae. This study is the first to investigate wildlife-associated bartonellae in Africa, adding support to their ubiquity and broad diversity and to the paradigm that the phylogenetic positions of the *Bartonella* species encountered today have been influenced by the geographical distribution of their hosts.

INTRODUCTION

Bartonella species are Gram-negative pleomorphic bacteria belonging to the α -*Proteobacteria* that are able to invade and persist within erythrocytes and thus chronically infect the blood of their hosts. Transmission between hosts is primarily mediated by haematophagous arthropods (Breitschwerdt & Kordick, 2000). Parasitism by bartonellae is widespread

among mammals; natural infections are highly prevalent and have been reported in many different species of insectivores, lagomorphs, rodents, artiodactyls, carnivores and primates. Although the genus currently contains only 20 recognized taxa, a large number of other isolates, mostly isolated from rodent communities worldwide, have been partially characterized (Birtles *et al.*, 1994; Kosoy *et al.*, 1997; Ellis *et al.*, 1999; Ying *et al.*, 2002; Holmberg *et al.*, 2003; Stevenson *et al.*, 2003). Sequence data for almost 200 isolates have been deposited in GenBank and comparative analysis of these suggests that most isolates cannot be reasonably accommodated within the current classification.

As bartonellae are phenotypically similar, characterization and differentiation of species is extremely reliant on genotypic and phylogenetic methods. DNA–DNA hybridization has been used in the definition of most species, although relatedness values very close to the 70 % threshold value have been reported (Kordick *et al.*, 1996). The usefulness of

Published online ahead of print on 16 April 2004 as DOI 10.1099/ijs.0.03033-0.

Abbreviations: ML, maximum likelihood; MP, maximum parsimony; SA, South African.

The GenBank/EMBL/DDBJ accession numbers for the *gltA* sequences of the *Bartonella* isolates reported in this study are AJ583112–AJ583136.

Transmission electron micrographs of cells of two novel isolates and a matrix of *gltA* dissimilarity scores are available as supplementary material in IJSEM Online.

comparative analysis of DNA sequence data to delineate *Bartonella* species has already been demonstrated (Lawson & Collins, 1996), and the application of this approach as the primary criterion for species definition has recently been proposed (La Scola *et al.*, 2003). Several genetic loci have been used for the characterization of *Bartonella* isolates (Houpikian & Raoult, 2001), but comparative analysis of citrate synthase gene (*gltA*) fragments remains the most commonly used approach for identifying bartonellae and inferring evolutionary relationships within the genus (Birtles & Raoult, 1996). Studies of inferred evolutionary relationships among *Bartonella* species have revealed that species parasitizing the same or similar host species are not necessarily closely related. However, phylogenetic reconstructions have demonstrated that clades comprising bartonellae isolated from species indigenous to Eurasia are divergent from clades containing isolates derived from animals indigenous to the Americas (Ellis *et al.*, 1999; Bown *et al.*, 2002).

Although much effort has been put into studying the biodiversity of bartonellae following their emergence as pathogens of humans and domesticated animals, fewer than 30 mammal species have been surveyed to date and thus it is likely that the true extent of variety within the genus is far larger than is currently recognized. In this study we have attempted to extend this exploration by studying bartonellae associated with small mammals endemic to South Africa. This is the first time that bartonellae associated with African wildlife have been examined and thus the results of this study should serve well in testing the validity of hypotheses concerning the basis of the divergence of bartonellae that have been derived from examination of isolates obtained from other continents.

METHODS

Sampling of animals. During 2000 and 2001, small terrestrial mammals were trapped live at sites in nine different Nature Reserves

of the Free State province, South Africa (Table 1). Trapping sites were selected in an attempt to sample as wide a diversity of animals as possible. The trapping schedule at each site involved setting 50 strategically placed, baited, Sherman traps over four consecutive days, with traps being checked each morning and evening. Trapped animals were identified and euthanized and blood was collected aseptically by cardiac puncture and stored frozen at -70 °C.

Isolation of *Bartonella* species and phenotypic characterization. A 100 µl aliquot of each blood sample was plated on to 10% horse blood-enriched Columbia agar. Plates were incubated at 35 °C in 5% CO₂ for up to 45 days. Plates were checked daily for bacterial growth, and colonies tentatively identified as bartonellae (small, round and grey-white) were passaged on to fresh plates. Putative bartonellae were identified on the basis of their microscopic appearance following Gram staining and their lack of biochemical reactivity when tested using API 20E strips (bioMérieux). To assess their ultra-structure, some isolates were prepared for transmission electron microscopy using standard methods. Briefly, bacteria were suspended in 2% phosphotungstic acid (pH 7.2), spread on a water surface and absorbed to Formvar-coated nickel grids. Samples were viewed under a Philips Tecnai 10 transmission electron microscope at 100 kV.

PCR and sequence analysis. Crude DNA extracts were obtained from isolates and a reference strain of *Bartonella henselae* (Houston-1^T) by boiling a heavy suspension of the organisms mixed (4:1) with a 20% (w/v) solution of Chelex 100 in distilled water. PCR-based amplification of *gltA* fragments was performed as previously described (Birtles & Raoult, 1996). Products were purified using a PCR Purification kit (Qiagen) and the nucleotide base sequence of each was determined commercially on both strands using the same primers as used in the PCR described above. For each amplification product, sequence data derived from each primer were compared, verified and combined using Align Plus 4 software (Scientific and Educational Software). Once ambiguities were resolved by repeating sequencing reactions, sequence similarity values between pairs of 698 bp *gltA* sequences from which primer sequences had been removed were determined using the same software.

The 698 bp sequences were then manually aligned with homologous *gltA* fragments using MacClade (Sinauer Associates). As many of the *gltA* sequences derived from *Bartonella* species and strains deposited

Table 1. Survey of *Bartonella* species infecting rodent species collected from Free State nature reserves, South Africa

Species: 1, *Aethomys namaquensis*; 2, *Mastomys natalensis*; 3, *Rhabdomys pumilio*; 4, *Otomys irroratus*; 5, *Pedetes capensis*; 6, *Malacothrix typica*; 7, *Rattus rattus*; 8, *Saccostomus campestris*; 9, *Suncus varilla*; 10, *Tatera leucogaster*. For each rodent species at each location, the number of animals from which a *Bartonella* isolate was obtained is given, with the total number of animals trapped in parentheses.

Nature reserve	Latitude and longitude	1	2	3	4	5	6	7	8	9	10	Total
Koppies	27° 15' S 27° 40' E		1 (1)									1 (1)
Sandveld	27° 40' S 25° 43' E		0 (1)			0 (14)		0 (1)	0 (1)		12 (19)	12 (36)
Willem Pretorius	28° 15' S 27° 28' E					0 (2)						0 (2)
Soetdoring	22° 25' S 26° 00' E		2 (2)									2 (2)
Caledon	29° 50' S 27° 40' E	0 (3)	0 (1)	1 (4)			0 (1)					1 (9)
Gariep	30° 35' S 25° 35' E		3 (7)	1 (3)	1 (2)							5 (12)
Iona	30° 29' S 25° 47' E	5 (5)	1 (1)	2 (2)								8 (8)
Tussen die Riviere	30° 30' S 26° 07' E	3 (8)	2 (2)						1 (1)	0 (2)		6 (13)
Naval Hill	30° 06' S 26° 14' E	3 (3)										3 (3)
Total		11 (19)	9 (15)	4 (9)	1 (2)	0 (16)	0 (1)	0 (1)	1 (2)	0 (2)	12 (19)	38 (86)

in GenBank were of a markedly shorter length, we also produced another data matrix (338 bp) to compare our data with all previously recognized *Bartonella* taxa. The two data matrices were analysed separately. Phylogenetic reconstructions were obtained by maximum-parsimony (MP) and maximum-likelihood (ML) methods as implemented by PAUP (version 4.0 beta 8) (Sinauer Associates) and by Bayesian analysis by using MrBayes (version 2.01) (Huelsenbeck & Ronquist, 2001; available at <http://morphbank.ebc.uu.se/mrbayes/>). MP heuristic searches for optimum trees were performed by swapping branches using the tree bisection reconnection algorithm, with all substitutions given equal weight. Branch support was assessed by 500 bootstrap replicates. The MP tree with the best likelihood score was used as the starting tree for the ML heuristic search. Nucleotide frequencies and transition/transversion ratios were estimated based on the MP tree via ML. Site-specific rates were estimated using a character partition by codon position. Bayesian Markov Monte Carlo analyses were performed by simultaneously running four independent chains for 500 000 replicates and by using a general time-reversible model, with relative site rates estimated by codon position. Trees were sampled every 100 iterations for a total of 5000 samples. Trees saved before the likelihood values stabilized were discarded from the final sample. A 50 % majority-rule consensus tree of the remaining trees was performed in PAUP and posterior probability values recorded for each branch.

Choice of sequences for inclusion in phylogenetic analyses.

The 338 bp alignment described above comprised 50 different sequences. These included 24 obtained from the South African (SA) rodents analysed in this study, 17 from representative strains of the *Bartonella* taxa with validly published names and nine from partially characterized *Bartonella* isolates. These nine were chosen either because they were found to share relatively high levels of sequence similarity with one or more of the sequences obtained from SA rodents (C5-rat and MR1) or because they were representatives of previously identified phylogenetic clades within the genus *Bartonella*; the provenance of R-phy2, C1-phy and R-phy1 has been detailed by Birtles *et al.* (1999) and their phylogeny explored in a study by Birtles & Raoult (1996), whereas the provenance and phylogenies of SH6397GA, SH6396GA, SH6537GA and PL7238NC have been detailed by Kosoy *et al.* (1997). The 698 bp alignment comprised 35 different sequences. Twenty-nine of these sequences belonged to the SA rodents or to other *Bartonella* taxa identified as sharing a specific relationship with the SA rodent strains after phylogenetic analysis of the 338 bp alignment. The remaining six sequences were included as outliers, against which the phylogeny inferred among the SA rodent isolates and their immediate sister taxa could be referred. The accession numbers for all sequences used in this study are given in Table 2 or adjacent to the *Bartonella* species name and/or strain designation in Fig. 1.

Calculation of frequency of inter-*gltA* sequence dissimilarity values. This work was attempted as an alternative approach to clarifying taxonomic relationships among the bartonellae recovered from SA rodents and other closely related isolates, and with the additional aim of further assessing the applicability of recently proposed criteria for the delineation of *Bartonella* taxa using gene sequence similarity values (La Scola *et al.*, 2003). Pairwise comparisons were determined among 29 partial (698 bp) *gltA* sequences obtained from members of the phylogenetically defined clusters I and II (see Fig. 2) using Align Plus 4. These clusters contained all SA rodent-associated isolates (25 sequences) and four other bartonellae (*Bartonella elizabethae*, *Bartonella grahamii*, C5rat and MR1). A total of 406 intersequence dissimilarity scores were obtained from these pairwise comparisons. The frequency of individual scores among these 406 was calculated and the distribution of frequencies examined.

RESULTS

Details of trapped rodents and culture success

A total of 86 animals were trapped during the study (Table 1). Overall trapping success was about 5 % and ranged from 0.5 % at Koppies to 18 % at Sandveld, suggesting that rodent abundance varied widely between sites. A total of 10 different species of small mammal were trapped. However, as 78 (91 %) of the animals belonged to five species, the remaining five species were each represented by only one or two individuals. The richness of trapped species also varied between sites, although this parameter was likely influenced by rodent abundance. Four sites, at all of which trapping success had been <1 %, yielded only one species. Between three and five species were trapped at the remaining five sites. The distribution of small mammal species also varied; five species were trapped exclusively at one site, although four of these were among those species represented by two or fewer representatives. Of the small mammals trapped in reasonable numbers, the Natal multimammate mouse, *Mastomys natalensis*, was found at seven sites, but the bushveld gerbil, *Tatera leucogaster*, was found at only one. Before euthanasia, all animals appeared healthy with no gross abnormalities.

The 86 blood samples yielded 38 cultures of putative bartonellae within 2 weeks of incubation. Colonial morphology was typical of primary *Bartonella* cultures, with small, off-white colonies appearing firm, dry and embedded. The number of c.f.u. varied from fewer than 10 to 'thousands' (per 100 µl sample). Subsequent passage was achieved for 32 of the 38 isolates (Table 2). The isolates lost were three obtained from *Aethomys namaquensis* trapped at Iona, one obtained from an *M. natalensis* trapped at Iona, one obtained from a *Rhabdomys pumilio* trapped at Caledon and one obtained from an *Otomys irroratus* (vlei rat) trapped at Gariep. All 32 successfully passaged isolates were confirmed as bartonellae on the basis of their microscopic appearance as weakly staining, Gram-negative, small, pleomorphic coccobacilli and on their biochemical inactivity when tested using an API 20E strip. Electron microscopy was performed on three isolates, AN-tr103, MN-ga6 and RP-io114 (chosen on the basis of their *gltA* sequence variation). Examination of electron micrographs revealed that all three isolates possess a typical Gram-negative, rod-shaped morphology. All three also appeared to possess lophotrichous flagella-like appendages and AN-tr103 possessed substantial polar tufts of pili-like appendages (see supplementary figures available in IJSEM Online).

The overall prevalence of infection was 44 %. No evidence of infection was found in three of the poorly represented small mammal species and, perhaps more interestingly, in the springhare, *Pedetes capensis*, for which 16 animals were tested (Table 1). All animals yielding bartonellae were, therefore, members of the rodent family Muridae. Among

Table 2. Provenance of the 32 successfully passaged *Bartonella* isolates

Common names of the host species are Namaqua rock mouse (*Aethomys namaquensis*), striped mouse (*Rhabdomys pumilio*), Natal multimammate mouse (*Mastomys natalensis*), bushveld gerbil (*Tatera leucogaster*) and pouched mouse (*Saccostomus campestris*).

Isolate	Host species	Trap location	GenBank accession no. (<i>gltA</i>)
AN-nh1	<i>Aethomys namaquensis</i>	Naval Hill	AJ583112
AN-nh2	<i>A. namaquensis</i>	Naval Hill	AJ583113
AN-nh3	<i>A. namaquensis</i>	Naval Hill	AJ583114
AN-tr1	<i>A. namaquensis</i>	Tussen die Riviere	AJ583115
AN-tr2	<i>A. namaquensis</i>	Tussen die Riviere	AJ583116
AN-io1	<i>A. namaquensis</i>	Iona	Identical to AN-nh1*
AN-io2	<i>A. namaquensis</i>	Iona	AJ583117
AN-tr103	<i>A. namaquensis</i>	Tussen die Riviere	AJ583118
RP-ga109	<i>Rhabdomys pumilio</i>	Gariep	AJ583119
RP-io111	<i>R. pumilio</i>	Iona	AJ583120
RP-io114	<i>R. pumilio</i>	Iona	AJ583121
MN-tr1	<i>Mastomys natalensis</i>	Tussen die Riviere	AJ583122
MN-so1	<i>M. natalensis</i>	Soetdoring	AJ583123
MN-so2	<i>M. natalensis</i>	Soetdoring	Identical to MN-so1
MN-ko1	<i>M. natalensis</i>	Koppies	AJ583124
MN-tr4	<i>M. natalensis</i>	Tussen die Riviere	AJ583125
MN-ga6	<i>M. natalensis</i>	Gariep	AJ583126
MN-ga8	<i>M. natalensis</i>	Gariep	AJ583127
MN-ga18	<i>M. natalensis</i>	Gariep	AJ583128
TL-sv1	<i>Tatera leucogaster</i>	Sandveld	AJ583129
TL-sv4	<i>T. leucogaster</i>	Sandveld	AJ583130
TL-sv6	<i>T. leucogaster</i>	Sandveld	AJ583131
TL-sv2	<i>T. leucogaster</i>	Sandveld	AJ583133
TL-sv3	<i>T. leucogaster</i>	Sandveld	AJ583134
TL-sv5	<i>T. leucogaster</i>	Sandveld	Identical to MN-so1
TL-sv7	<i>T. leucogaster</i>	Sandveld	AJ583135
TL-sv8	<i>T. leucogaster</i>	Sandveld	Identical to TL-sv1
TL-sv9	<i>T. leucogaster</i>	Sandveld	AJ583136
TL-sv10	<i>T. leucogaster</i>	Sandveld	Identical to TL-sv9
TL-sv15	<i>T. leucogaster</i>	Sandveld	Identical to TL-sv1
TL-sv16	<i>T. leucogaster</i>	Sandveld	Identical to MN-so1
SC-tr1	<i>Saccostomus campestris</i>	Tussen die Riviere	AJ583132

*Sequences found to be identical to others encountered earlier in the study were not submitted to GenBank.

the well-represented species, infection prevalences were similar, ranging from 44 % in the striped mouse, *R. pumilio*, to 63 % in *T. leucogaster* (Table 1). Overall infection prevalence and host-specific infection prevalences also varied among sites (Table 1).

***gltA* sequence analysis**

Partial *gltA* fragments of 698 bp were obtained for all 32 successfully passaged isolates. Two sets of comparative sequence analyses were performed on these new data. First, all sequences were shortened to 338 bp to allow their comparison with those available for all previously characterized *Bartonella* species (as available data for some of

these species were limited to this length of sequence). Comparison of these data demonstrated that the isolates yielded 24 different sequences, all of which were distinct from the *gltA* sequences of the recognized *Bartonella* species or other partially characterized strains. The *gltA* sequences most similar to those of the SA rodent-associated bartonellae were those of *B. elizabethae*, *B. grahamii* and *Bartonella tribocorum*, but these were at least 4 % dissimilar.

The second set of comparative sequence analyses was performed using full-length (698 bp) *gltA* fragments from the SA rodent-associated isolates together with those available from other *Bartonella* species and strains. Unsurprisingly, some of the full-length sequences were indistinguishable

Fig. 1. Phylogenetic tree inferred using a Bayesian approach from an alignment of 338 bp *gltA* fragments obtained from the SA rodent-associated bartonellae, representatives of the established *Bartonella* taxa and other partially characterized *Bartonella* isolates. Significant bootstrap values are indicated at relevant nodes.

from one another. Sequences from isolates TL-sv1, TL-sv8 and TL-sv15 were the same as one another, as were those from isolates TL-sv9 and TL-sv10, isolates TL-sv5, TL-sv16, MN-so1 and MN-so2, and isolates AN-nh1 and AN-io1 (Table 2). Thus, 25 different sequences were obtained; the *gltA* sequences of isolates AN-nh1 and AN-nh3, which were identical when compared over 338 bp (above), differed when compared over 698 bp. None of the 25 sequences were identical to any other *gltA* sequence in GenBank and, as observed for the 338 bp sequences, they shared highest similarity (96 %) with 698 bp *gltA* fragments of *B. elizabethae* and *B. grahamii*. However, among the different *gltA* sequences obtained from the SA rodent-associated bartonellae, similarity values ranged from 91 to 99 %.

Phylogeny

The 338 bp alignment contained 105 parsimony informative characters. The MP heuristic search recovered a single most parsimonious tree (length 444; CI=0.480; RI=0.687; not shown). The Bayesian 50 % majority rule consensus tree of the 4800 retained samples is shown in Fig. 1, from which it was apparent that the SA rodent-associated bartonellae shared a strongly supported, discrete cluster within

the radius of the genus *Bartonella* with the species *B. grahamii*, *B. elizabethae* and *B. tribocorum*.

The 698 bp alignment contained a total of 160 parsimony informative characters. The MP heuristic search recovered a single most parsimonious tree (length=474; CI=0.605; RI=0.790) (Fig. 2). Isolates R-phy2, R-phy1, C1-phy and *Bartonella vinsonii* subsp. *arupensis*, *B. vinsonii* subsp. *vinsonii* and *Bartonella taylorii* were used as outgroups, because bootstrapping separated these sequences from all the others in 100 % of the replicates. The ML heuristic search was completed after 225 368 rearrangements and produced a total of 16 ML trees. Because the topologies of the ML tree with the best ML score ($-\ln L=3377.44$) and of the Bayesian 50 % majority-rule consensus tree were perfectly identical, we have only shown the Bayesian reconstruction (Fig. 2). This tree was obtained after the 130 trees, sampled before the likelihood values became stable, were eliminated.

The architecture of the MP and Bayesian-derived reconstructions presented in Fig. 2 were very similar to one another and to those inferred from the 338 bp alignment (Fig. 1). Indeed, in Fig. 2, the clusters resolved by the two methods of analysis were the same with the exception of

Fig. 2. Phylogenetic trees inferred using a Bayesian (left-hand side) and an MP (right-hand side) approach from alignment of 698 bp *gltA* fragments obtained from the SA rodent-associated bartonellae, representatives of closely related *Bartonella* taxa and other partially characterized *Bartonella* isolates. Significant bootstrap values are indicated at relevant nodes. Phylogenetic clusters, as defined in the text, are indicated by dashed boxes.

those containing RP-io111, RP-io114 and RP-ga109. Inference of phylogenetic relationships from the 698 bp indicated that the SA rodent bartonellae formed two discrete clusters. Cluster I contained bartonellae infecting *A. namaquensis*, *T. leucogaster* and *Saccostomus campestris* together with bartonellae that are associated with brown rats worldwide (*B. elizabethae*, C5-rat and MR1) and European woodland rodents (*B. grahamii*). Cluster II contained exclusively SA rodent-associated bartonellae, including all isolates from *M. natalensis* or *R. pumilio* (Table 2) together with some of the isolates obtained from *A. namaquensis* and *T. leucogaster* (Table 2). Within this cluster, most of the isolates obtained from *M. natalensis* grouped together (with the exception of MN-tr4). Although the three isolates obtained from *R. pumilio* did not reliably group together, they were all outliers within cluster II, demonstrating clear divergence from the other members of the cluster (Fig. 2).

Distribution of inter-*gltA* sequence dissimilarity score frequencies

The 406 pairwise *gltA* sequence dissimilarity scores determined among cluster I and II members are presented in a

matrix (see supplementary table available in IJSEM Online) and ranged from 1 to 63 bp. The frequencies of different dissimilarity scores within this range varied and examination of this variation (Fig. 3) suggested that these frequencies fell into a bimodal distribution made up of a smaller mode of frequencies between 1 and 15 bp dissimilarity and a far larger mode of frequencies between approximately 20 and 63 bp dissimilarity.

DISCUSSION

This is the first published study confirming the presence of bartonellae in rodents endemic to Southern Africa and the first to investigate the diversity of organisms associated with these infections across the entire African continent. The presence and dynamics of *Bartonella* infections of *Psammomys obesus* (fat sand rats) in Tunisia have been previously reported (Fichet-Calvet *et al.*, 2000), but the identities of these bartonellae have yet to be published. Although our observations in relation to rodents are new, several studies reporting on *Bartonella* species currently recognized as human pathogens in Africa have been

Fig. 3. Bimodal distribution of intersequence dissimilarity score frequencies, as measured by pairwise comparison of 698 bp *gltA* fragments of the SA rodent-associated bartonellae and other representatives of the genus *Bartonella*. The previously proposed 96% threshold value (La Scola *et al.*, 2003) that equates to a dissimilarity score of 28 bp is indicated on the distribution. Superimposed on the distribution are intersequence dissimilarity scores between representatives of several established *Bartonella* taxa. Their addition is relevant to the discussion and demonstrates the range of scores obtained when taxa are compared.

published. Evidence of *B. henselae* infections in cats and humans has been reported (Childs *et al.*, 1995; Raoult *et al.*, 1996; Kelly *et al.*, 1996, 1998; Pretorius *et al.*, 1999; Frean *et al.*, 2002) and a role for *Bartonella quintana* as a significant cause of louse-borne disease on the continent has also been demonstrated (Raoult *et al.*, 1998).

The detection of bartonellae in SA rodents is not at all unexpected given that infections have been encountered in all reported surveys of rodent populations elsewhere in both the Old and New Worlds. Communities of rodents and insectivores in Europe (Birtles *et al.*, 1994; Holmberg *et al.*, 2003), Asia (Ying *et al.*, 2002) and North and South America (Kosoy *et al.*, 1997; Birtles *et al.*, 1999) have all been shown to maintain bartonella infections. The overall prevalence of infections observed in SA small mammals (44%) is similar to that reported elsewhere in the world.

The pattern of clustering of the SA rodent bartonellae is noteworthy. Cluster I includes members of a previously recognized cluster of strains associated with species of Old World rats and mice (Rodentia, Muridae, subfamily Murinae) and Old World-inhabiting species of arvicoline voles (Rodentia, Muridae, subfamily Arvicolinae). Our observations extend the host range associated with this cluster to include the Murinae genus *Aethomys*, the Gerbillinae genus *Tatera* and the Cricetomyinae genus *Saccostomus* (pouched mice). As cluster II appears to represent a novel evolutionary lineage within the genus, no *Bartonella* isolate of African origin shares any specific evolutionary relationship with *Bartonella* strains associated with hosts endemic to the New World. This observation further supports the hypothesis that bartonellae associated with hosts native to the Old World are phylogenetically distinct from those associated with host species native to the New World (Ellis *et al.*, 1999).

Examination of sequence similarity data and the inferred

phylogenies indicated that some degree of host-specificity among the strains examined exists. Cluster I included three groups of closely related isolates that were obtained from a single host species – two of these groups exclusively contained isolates infecting the only Gerbillinae species studied, *T. leucogaster*, and one group was associated with *A. namaquensis*. Cluster II included one group dominated by isolates infecting *A. namaquensis*, one group made up of isolates exclusively associated with *M. natalensis* and a less-well-defined group made up of all three isolates recovered from *R. pumilio*. However, against this background of host preference, there was a clear indication that different host species are susceptible to infection by the same *Bartonella* species, with, for example, isolates obtained from *M. natalensis* and *T. leucogaster* yielding the same *gltA* sequence and an isolate from *M. natalensis* lying within the cluster II group dominated by isolates from *A. namaquensis*. Imperfect host specificity of *Bartonella* species, particularly those associated with rodents, has been frequently observed (Birtles *et al.*, 1994; Kosoy *et al.*, 1999; Holmberg *et al.*, 2003). From the discussion above, it is also apparent that some host species, for example *T. leucogaster*, are susceptible to infection by phylogenetically distinct strains of *Bartonella*; this phenomenon has also been previously observed, with, for example, cats being considered hosts to three *Bartonella* species (Droz *et al.*, 1999). Intriguingly, no evidence of bartonella parasitism was found in the springhare, *Pedetes capensis*, despite a reasonable-sized sample (16). Although some other mammalian species have also been reported to be free of bartonellae (Heller *et al.*, 1999), *Pedetes capensis* is the first rodent species in which no evidence of infection can be found. Springhares are the sole members the family Pedetidae within the order Rodentia and as such are evolutionarily quite distinct from the other rodents sampled in this study.

In the context of the current discussion of the role of sequence similarity data in the definition and delineation

of bacterial species, the distribution of frequencies of intersequence variation observed among the 29 698 bp *gltA* fragments obtained from the SA rodent-associated bartonellae and four other *Bartonella* strains within cluster I and II is noteworthy. Bacteria fall into clusters of closely related organisms based on sequence similarity of shared protein-encoding genes, such that the sequence divergence within a cluster is far less than that between strains of different clusters. Theoretical work has suggested that each of these clusters corresponds to an ecologically distinct population (Palys *et al.*, 1997) and empirical support for this theory has been published (Palys *et al.*, 2000). As the clusters recognized by sequence similarity comparisons have an ecological relevance, it is rational to propose that measurement of this parameter should be a major criterion in demarcating bacterial taxa (Lan & Reeves, 2001). Our data generally support such an approach, with the frequencies of inter-*gltA* sequence variation among our isolates forming a bimodal distribution. The first mode comprised variations between specifically related strains (ecotypes), whereas the second mode comprised variations between strains belonging to ecologically distinct populations of bartonellae. When inter-*gltA* sequence variations between strains designated as belonging to the polyphasically established *Bartonella* taxa (both species and subspecies) were superimposed on to this distribution, they were observed to lie exclusively within the second mode (Fig. 3), thereby fulfilling the definition of separate ecotypes. Although this observation suggests concurrence between traditionally defined taxonomic delineations and those revealed by gene sequence similarity assessments, the fact that intersequence variation values between *Bartonella* subspecies lie within the same continuum as those observed between *Bartonella* species indicates that subspecies cannot be reliably delineated using a sequence similarity-based approach. As a result of this failure, the ecological relevance of the subspecies demarcation among *Bartonella* taxa must be called into question.

Of particular interest were the handful of sequence variation values between 9 and 15 bp. These values may represent outliers within the broadening genetic spectrum of an ecotype that is generated between periodic selection events (Cohen, 2002), but may also result from recombination of alleles from different ecotypes. Examination of the source of these intermediate sequence variation values indicated that they were derived from comparison of AN-nh2 *gltA* with the very similar *gltA* sequences of AN-nh1 and four other isolates that formed a monophyletic group (see Fig. 2). The distribution of the 12 (synonymous) nucleotide differences within the 698 bp alignment of AN-nh1 and AN-nh2 *gltAs* indicated that 7/12 lay among the first 106 bp and 4/12 lay among the final 43 bp. This distribution was markedly different from that observed in pairwise *gltA* alignments of members of different ecotypes, in which differences were more evenly dispersed. Furthermore, the 5' and 3' extremities of the AN-nh2 *gltA* sequence were very similar to the *gltA* sequences of members of the monophyletic cluster containing MN-tr1 and eight other

isolates (Fig. 2). These observations suggested that recombination may have played a role in shaping the identity of AN-nh2 *gltA*. The role of intraspecies recombination in the generation of genetic diversity among *B. henselae* strains has been demonstrated (Iredell *et al.*, 2003), and an extension of the multilocus sequence typing approach used therein to examine the importance of inter-*Bartonella* species (or interecotype) recombination events would be valuable.

La Scola *et al.* (2003) recently proposed cut-off sequence similarity values for *Bartonella* species definition, suggesting that an isolate could be considered as being a novel species if a 327 bp *gltA* fragment shared <96.0% sequence similarity with those of species with validly published names. This cut-off equates to 28 bp of intersequence variation between the 698 bp *gltA* fragments we examined, well above the lower limit of interecotype variation observed in Fig. 3, and thus it would appear that its application to species delineation is supported by our data. However, paradoxically, our data also demonstrate the shortcomings of using an approach based solely on sequence similarity assessments for species definition. Comparison of the *gltA* sequence similarities between TL-sv7 and the three isolates AN-tr1, AN-tr2 and AN-tr103 yielded differences of 29, 26 and 28 bp, respectively. Thus, by applying the criterion described above, TL-sv7 is a separate species from AN-tr1 and AN-tr103 but its relationship with AN-tr2 is unresolved. However, phylogenetic analysis of these sequences (Fig. 2) indicated that AN-tr1, AN-tr2 and AN-tr103 formed a closely related monophyletic group that was divergent from the branch carrying TL-sv7. Thus, as proposed elsewhere (Lan & Reeves, 2001), a more sensitive ecologically relevant delineation of *Bartonella* species could be achieved using a combination of sequence similarity analysis and phylogenetics. However, to reiterate the discussion above, such an approach would benefit greatly from a multilocus approach.

ACKNOWLEDGEMENTS

We thank J. Watson from the Department of Environmental Affairs and Tourism of the Free State province for his help with capturing and identification of rodent species. Thanks also to Dr A. Radford for useful discussions about the exploitation of sequence similarity values for delineating bacterial ecotypes. This study was partially funded by the University of the Free State, Bloemfontein, South Africa. R.J.B. is supported by a Wellcome Trust Medical Microbiology Fellowship. Part of this work was presented at the American Society for Rickettsiology–*Bartonella* as an Emerging Pathogen Group, Joint Conference, Big Sky, Montana, 18–22 August 2001 (abstract 131).

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