in 1.1% agarose (SeaKem Gold; Cambrex Bio Science) in 0.5 × TBE buffer in a GenNavigator System apparatus (Amersham Biosciences, Uppsala, Sweden) at 14 °C and 5.6 V/cm, for a total of 65 h with switch times ramped from 5 to 150 s. The gels were stained with ethidium bromide (0.5 μg/mL) at room temperature for 1 h, destained in 0.5 × TBE buffer for 30 min, and visualized under UV light. The sizes of the fragments were estimated using pulsed-field gel electrophoresis (PFGE) λ-ladder, Low Range PFG marker, and Yeast Chromosome PFG marker (New England Biolabs).

Isolation of phage particles, extraction of phage DNA and gel electrophoresis of phage DNA

Phage particles were isolated and phage DNA was extracted and amplified with the REPLI-g Mini kit (Qiagen, Valencia, CA) as described previously (Berglund *et al.* 2009). PFGE was performed as described above with the following modifications: phage particles in PBS were directly moulded into plugs, no digestion was performed, and the DNA fragments were separated by electrophoresis at 5 V/cm for a total of 29 h.

Nucleotide sequence and microarray accession numbers

The novel sequences have been submitted to EMBL with accession numbers shown in Table S1 (Supporting Information). The microarray data have been deposited in the ArrayExpress database of the European Bioinformatics Institute under the accession numbers A-MEXP-1576 for the array design and E-TABM-875 for the experimental data.

Results

We have analysed 26 strains of *B. grahamii* isolated from mice and voles of four genera (*Apodemus, Myodes, Microtus* and *Arvicola*) that were captured in seven countries in North America, Europe and Asia (Table 1). These strains were selected from a much larger collection, in order to represent most of the diversity. Studies of *Bartonella* prevalence in rodent populations in continents that are not included in our study (Africa, Australia and South America) have not yielded any *B. grahamii* isolates (Birtles *et al.* 1999; Pretorius *et al.* 2004; Gundi *et al.* 2009). We thus believe that we have a good representation of the known diversity of *B. grahamii*. The relationships of the 26 strains were inferred by sequence analysis of eleven loci, their gene contents were investigated by microarray hybridizations using the sequenced *B. grahamii* strain as4aup as reference and the genome sizes of all strains were estimated by pulsed-field gel electrophoresis.

Geographic population structure

We first sequenced eleven loci (three genes and eight intergenic regions) in all strains, with the number of alleles ranging from 9 to 16 per locus (Table 2). The alignments for all loci were concatenated to a total length of *c.* 7 kb, and maximum likelihood phylogenetic analysis revealed a clustering of *B. grahamii* strains according to geographic origin (Fig. 1). The tree had two major clades: one containing the Asian strains and the other the European and American strains (Fig. 1).

A closer inspection revealed that the Asian strains were divided into a Japanese and a Chinese group, and that all Canadian strains formed a single clade, as did also all European strains. One European subclade, here called Europe 1, contained the Swedish, Russian and two British strains (V2 and WM11) and the other subclade, here called Europe 2, the remaining British isolates. The bootstrap support for each of the described groups was 100%. Finally, the results indicated that the U.S. strain Mo12658sd represents the earliest diverging branch within the European–American clade, with 92% bootstrap support for the cohesion of all other strains to the exclusion of this isolate. As some intergenic regions contained long indels, we also inferred maximum likelihood phylogenies based on only the concatenated genic regions (Fig. S1, Supporting Information) and from the original alignment after removal of all sites with gaps (Fig. S2, Supporting Information). The major clades described above were highly supported also in these two trees.

For comparison with the previously described diversity of *B. grahamii* we inferred a phylogeny of the *gltA* gene, which includes published sequences from other countries (Fig. S3, Supporting Information). Because of the low level of sequence information contained in this gene, the bootstrap support values for the different clades were low, and we could not root the tree reliably. Despite these limitations, the tree reveals that there are no major clades within *B. grahamii* that are absent from our data set. However, the diversity within Europe is larger than revealed by the isolates included in this study, with two strains from Greece and Poland clustering with the other European strains whereas two others cluster with the American strains.

Recombination is extensive in the Asian strains

We observed a high degree of sequence divergence among the Chinese and Japanese strains, with an average pairwise sequence identity of only 95.9% excluding sites with gaps (Table S2, Supporting Information). In contrast, the average sequence identity between European and American strains was as high as 98.2% (Table S2, Supporting Information). Additionally, large inser-

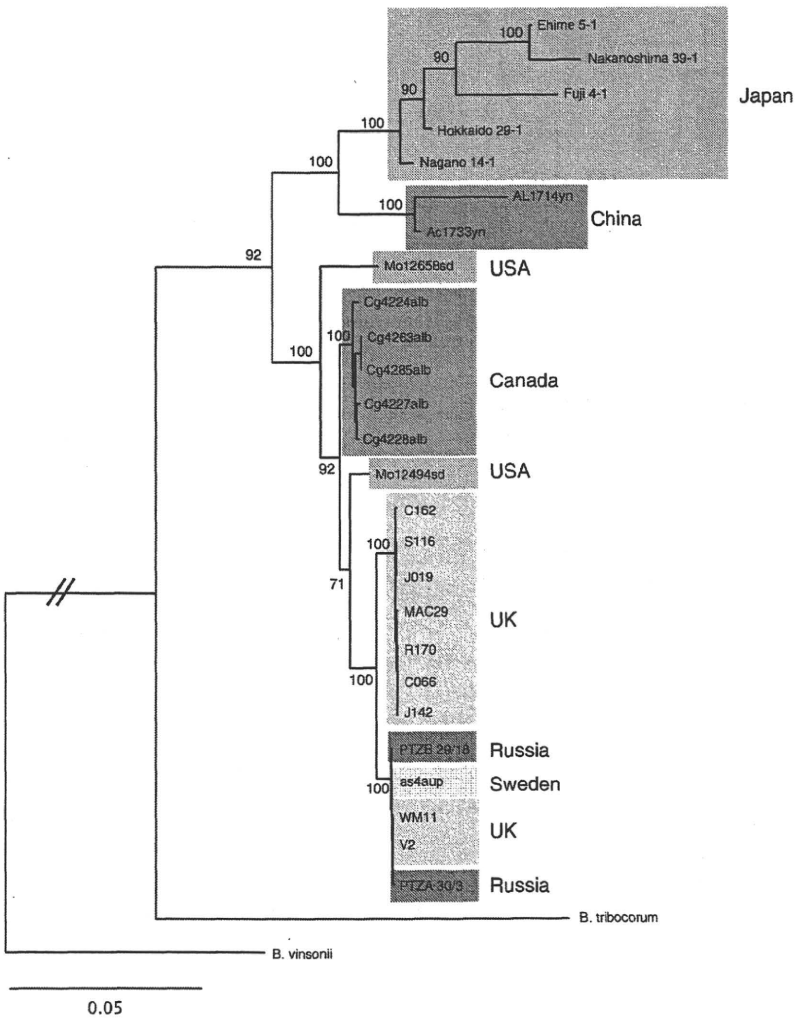

Fig. 1 Phylogenetic tree inferred from a concatenated alignment of all sequenced loci. Branch lengths are according to maximum likelihood, and bootstrap support values for the major clades are shown. Strains are colour-coded according to geographic origin. The branch length of *Bartonella vinsonii* is not according to scale.

tion–deletion events were identified in three intergenic regions in the Asian strains: Fuji 4-1 and AL1714yn shared a deletion in locus 9, Nagano 14-1 and Nakanoshima 39-1 had an insertion in locus 4, and all Asian strains had an insertion in locus 11. Both insertions included short segments with similarity to phage genes in *B. tribocorum* (Btr_0462, E < e^{-8} and Btr_0221, E < e^{-6}, respectively). In the European–American strains, no large indels were observed.

To investigate whether each sequenced region supported the same tree topology, we inferred a phylogenetic tree for each locus, and computed a super-network from these trees using SplitsTree4. Although the network confirmed that the population structure of the *B. grahamii* strains is mostly a function of geography, conflicts were abundant within the Asian clade (Fig. 2). Manual inspection of each tree showed that the conflicts were often well supported by bootstrap values (Fig. 3), indicative of recombination between the Asian strains. Recombination also appears to occur between *Bartonella* species, exemplified by the clustering of the Japanese strain Fuji 4-1 and *B. tribocorum* with a bootstrap support value of 92% in locus 5 (Fig. S4, Supporting Information). For comparison, we also computed a super-network of a MST data set from *B. henselae* (Li *et al.* 2006), which revealed no conflicts between trees (data not shown).

Recombination events among Asian strains were also predicted within five of the sequenced regions (Table S3, Supporting Information), by at least three different algorithms in RDP3 ($P < 0.05$). The predicted recombined segments spanned *c.* 100–600 bp and occurred in genes, spacers as well as across borders of genes and spacers (Table S3, Supporting Information). Of the five loci in which recombination events were detected, four were associated with phage gene remnants (loci 3, 4, 10 and 11). No remnants of phage genes were identified in any of the six loci at which no recombination events were detected.

For comparison, we also computed a super-network and applied RDP3 to the *B. grahamii* MLST data set from (Inoue *et al.* 2009). The results showed that

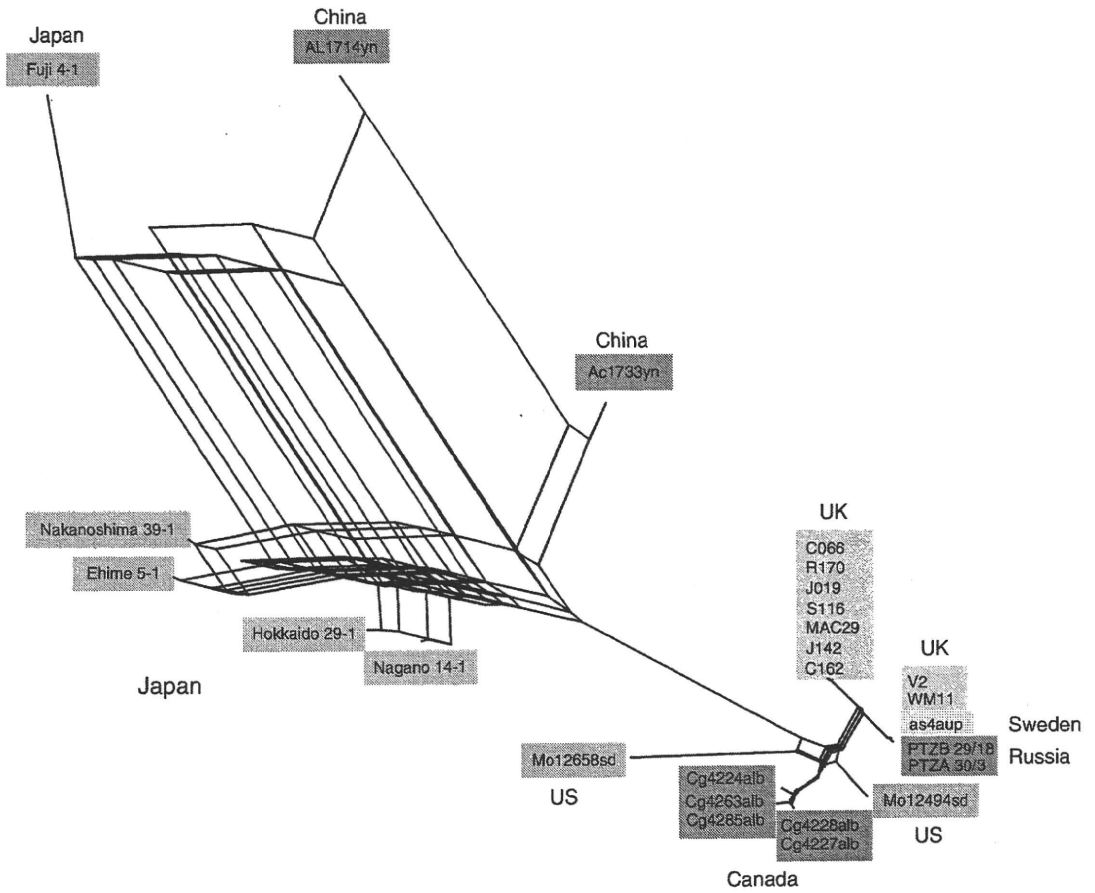

Fig. 2 Super-network of the analysed *Bartonella grahamii* strains, based on the individual trees for each sequenced locus. Conflicts between individual tree topologies, represented as rectangular boxes in the network, are abundant in the Asian group. Strains are colour-coded according to geographic orgin.

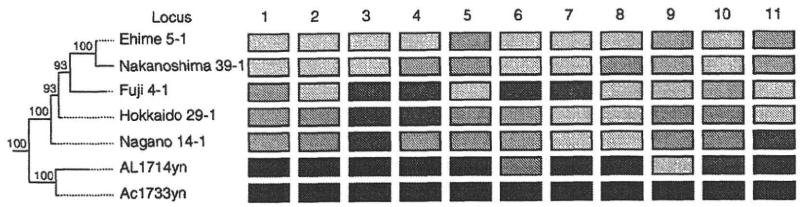

Fig. 3 Schematic representation of highly supported subclades of Asian strains in individual phylogenies of each sequenced locus. The phylogeny of the Asian strains (from Fig. 1) is shown to the left. Each locus (numbered as in Table 2) is represented by one column to the right, and clades in the phylogeny of that locus that are identical or that have at least 85% bootstrap support, are marked in the same colour. Groups that are coloured in different shades of green or purple indicate that these subclades also cluster together with at least 85% support.

conflicts were abundant in the Asian group, and recombination events were detected in three of six genes (*ribC*, *rpoB* and *ftsZ*) (Table S4, Supporting Information). The relative contribution of recombination vs. nucleotide substitution (the *r/m* ratio) in this data set was estimated to 1.7, which means that the probability that a sequence polymorphism is a result of recombination is slightly higher than the probability for nucleotide substitutions. Altogether, these results suggest that recombination is frequent in the Asian population of *B. grahamii*.

To examine possible influences of recombination on the tree topology, and whether the high bootstrap support values in Fig. 1 underestimates the uncertainty, we inferred a phylogenetic tree of the complete data set using ClonalFrame, which takes recombination into

account when building trees. The analysis resulted in a tree with the same major clades, all with high posterior probabilities, as the maximum likelihood tree in Fig. 1 (Fig. S5, Supporting Information). The main difference between the topology of our phylogenetic tree and that inferred by Inoue *et al.* is that all Japanese strains grouped together in our tree whereas they were separated into two groups in theirs (Inoue *et al.* 2008; Inoue *et al.* 2009). Interestingly, in a phylogenetic tree inferred from only the three genes where no recombination events were detected in Inoue's data set, all Japanese strains clustered together in one clade (data not shown). In our data set, removal of the recombined loci did not influence the topology. It thus appears as if the population structure of *B. grahamii* can be reliably inferred if enough loci with enough variation are sequenced, even though individual trees may present conflicts.

CGH data reveal gene content variations in genomic islands and plasmids

Pulsed-field gel electrophoresis (PFGE) with the *Not*I restriction enzyme revealed a great variability in genome structure, with 25 distinct patterns in the 26 strains (Fig. S6, Supporting Information). The genome sizes were estimated to range from less than 2 Mb in the Canadian strains Cg4263alb and Cg4285alb to more than 2.5 Mb in Hokkaido 29-1 (Fig. S6, Supporting Information).

We analysed differences in gene content by comparative genome hybridizations to a microarray designed based on the genome of *B. grahamii* strain as4aup from Sweden. The array contains 4438 probes, which cover 96% of the 1768 annotated genes in this genome. The results showed that most variations in gene content are concentrated to the previously described genomic islands, including the *badA* region in BgGI 1, the prophage regions in BgGI 2 and BgGI 4, the membrane proteins in BgGI 3, the phage-related BgGI 5, and the Vbh T4SS in BgGI 16 (Fig. 4). Variability was also recorded in the so-called *fha*-repeat, which is present in the islands BgGI 4, BgGI 7, BgGI 10, BgGI 11 and BgGI 14 (Fig. 4). The plasmid pBGR3, which encodes a copy of the Vbh T4SS, showed a highly scattered distribution pattern, and appeared to be present in at least one strain from each continent (Fig. S7, Supporting Information). Major deletions were observed in the Canadian strains Cg4263alb and Cg4285alb, in agreement with the small genome size estimate from the PFGE.

In the Asian strains, low hybridization signals were observed for many more genes, including core genes (Fig. 4). To check whether the low signal in these genes could be an effect of high levels of sequence divergence relative to the Swedish reference genome rather than gene loss, we estimated the sequence conservation across the genome by calculating the percentage identity of each probe to the genome of *B. tribocorum*. The results confirmed that many of the core genes that showed a low hybridization signal in the Asian strains are among the most divergent between *B. grahamii* and *B. tribocorum* (Fig. 4).

Clustering patterns of the European–American strains by CGH data

To examine the extent to which gene contents correlate with the population structure as inferred from the sequence data, we divided the genome into regions of present or absent ($M \leq -2$) probes, and inferred a maximum parsimony tree from this data (Fig. 5). Because of their high sequence divergence, the Asian strains were excluded from this analysis. With the exception of the British strain C162, which clusters with the Canadian strains, the resulting tree topology is largely in agreement with the sequence-based tree topology, with a major division between American and European strains, and the same two subgroups of European strains.

As the sequenced strain belongs to Europe 1, it is not surprising that the strains with the fewest number of losses belong to this group. These strains are unique in that the *badA* region showed no decrease in signal, and the few observed losses for this group were mostly confined to phage genes and the *fha*-repeat. In contrast, strains belonging to Europe 2 displayed decreased hybridization signals at several sites, including the *badA* region, BgGI 3, the repeated genes in the *trw* locus for a T4SS as well as in the *bep* genes located immediately downstream of the *virB* locus for another T4SS. Partial losses of the *fha*-repeat were observed in most Europe 2 strains (Fig. S8, Supporting Information).

In comparison, the Canadian strains showed a much more extensive loss of genes, mainly within the *fha*-repeat and in prophage I (Fig. 4). An almost complete loss of the *fha*-repeat was also found in the British strain C162, and this might be the reason why this strain clusters with the Canadian strains. The Canadian strain Cg4228alb is the earliest diverging in the Canadian clade and atypical in that it only showed partial losses of the *fha*-repeat, thus resembling the Europe 2 isolates over these segments (Fig. S8, Supporting Information). The two U.S. strains, which did not cluster together in the sequence-based tree, also displayed major differences in terms of gene content. Mo12658sd was most divergent on the sequence level and also showed more gene loss, including, for example, BgGI 3 and BgGI 5.

Two sets of strains that were identical in the sequence analysis (Cg4263alb and Cg4285alb; C066, J142 and R170) also clustered together in Fig. 5. Thus, the relationships

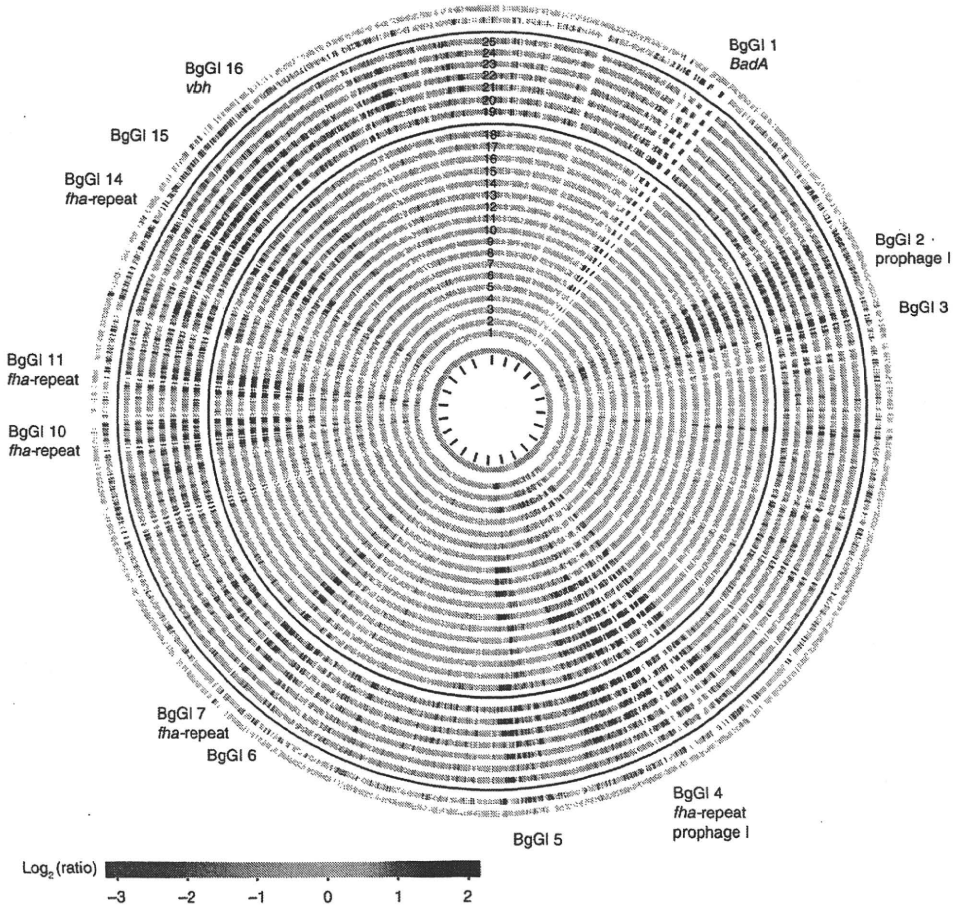

Fig. 4 Circular representation of the microarray data for the *Bartonella grahamii* chromosome, ordered according to the genome position in the sequenced strain. The innermost circle shows the genomic islands in magenta and prophages in yellow. The black lines within this circle indicate the genome position, with 100 kb between each line. Each other circle, except the two outermost, shows the microarray results for one strain, with the colour corresponding to the hybridization signal relative to as4aup (red for lower and blue for higher signal). The strains are: 1, V2; 2, WM11; 3, PTZA 30/3; 4, PTZB 29/14; 5, J142; 6, C066; 7, R170; 8, J019; 9, S116; 10, MAC29; 11, C162; 12, Cg4228alb; 13, Cg4224alb; 14, Cg4227alb; 15, Cg4263alb; 16, Cg4285alb; 17, Mo12494sd; 18, Mo12658sd; 19, Ac1733yn; 20, AL1714yn; 21, Ehime 5-1; 22, Nakanoshima 39-1; 23, Nagano 14-1; 24, Fuji 4-1; 25, Hokkaido 29-1. The second outermost circle shows the nucleotide identity in a global alignment of each probe to the *Bartonella tribocorum* genome, with probes with less than 75% identity in red. The 75% cutoff is based on previous control hybridizations with *B. henselae* and *B. quintana* (Lindroos *et al.* 2005). The outermost circle shows the predicted presence (grey) (Blastp, cutoff $E < e^{-10}$) or absence (red) of each *B. grahamii* gene in the genome of *B. tribocorum*. The black circles show the border between European-American strains, Asian strains and predictions from the *B. tribocorum* genome.

of strains as inferred by their deletion–insertion patterns were broadly consistent with the sequence-based tree topology, and both support relationships governed by geographic vicinity. There were however several examples of independent gains or losses of genes in strains from different countries, for example, the *fha*-repeat (lost from the British strain C162 and most Canadian strains), BgGI 3 (at least partial losses in all Europe two strains except J019 and all American strains except Mo12494sd) and BgGI 5 (lost from the British isolate MAC29 and the US isolate Mo12658sd). Most of the variably present genes are repeated in the genome of the sequenced strain, and the lower microarray signal can be due either to a complete loss, a lower copy number or a high sequence divergence.

Duplication and replication of phage-related genomic islands

Several strains displayed increased signals in prophage I and around BgGI 15, where the putative origin of run-off replication is located. The island BgGI 5, which contains phage genes homologous to those that encode the newly described *Bartonella* GTA, also displayed

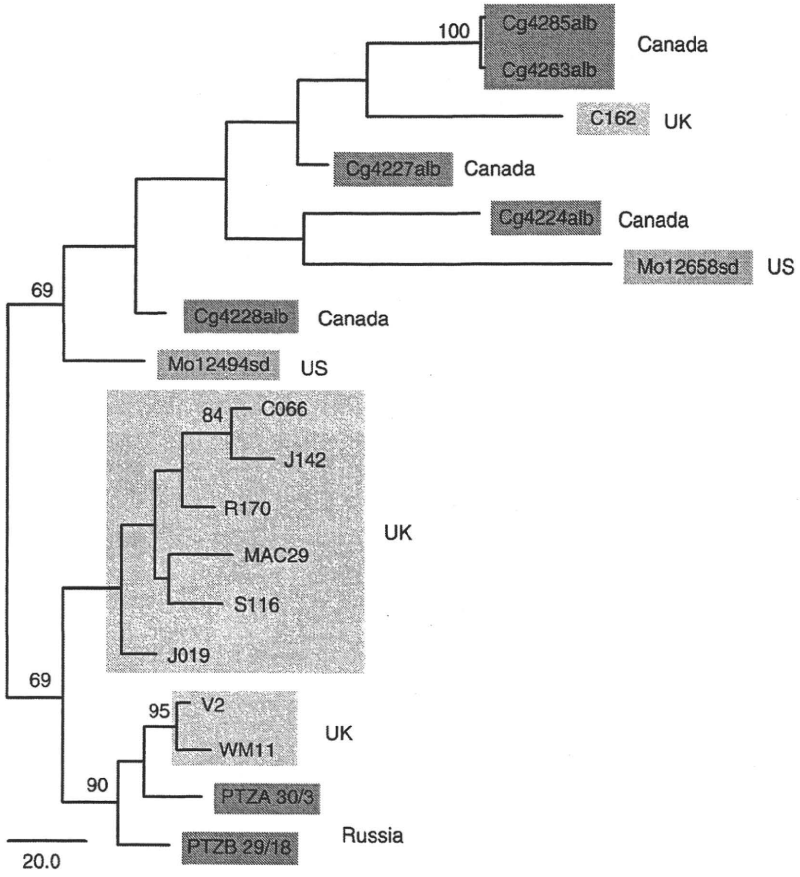

Fig. 5 Maximum parsimony tree of European and American strains based on microarray data. Bootstrap support values over 65 are shown. Strains are colour-coded according to geographic orgin.

increased signal in several strains (Fig. 5). To investigate whether this island is replicated and packaged into phage particles we isolated phage DNA from the strains Cg4228alb and PTZA 30/3 and hybridized to the array against total bacterial DNA from the same strain. The results revealed run-off replication and a slight over-representation of prophage I in the phage DNA, but no increase in signal in BgGI 5. Gel electrophoresis of the phage DNA also did not reveal any other bands than the previously observed 14 and 45 kb bands (data not shown). These observations suggest that the increase in signal is more likely due to duplication(s) than replication of BgGI 5. There was no sign of gene duplication in Hokkaido 29-1, which had an estimated genome size of more than 2.5 Mb, suggesting that additional genes are present in this strain.

Discussion

Influence of host ecology and population structures on the genomic diversity of Bartonella populations

Our study of the genomic diversity in a global *B. grahamii* population revealed a stronger geographic pattern and higher levels of sequence and gene content divergence than has been observed previously in *B. henselae* and *B. quintana* (Iredell *et al.* 2003; Foucault *et al.* 2005; Li *et al.* 2006, 2007; Lindroos *et al.* 2006; Arvand *et al.* 2007). The strong geographic pattern of *B. grahamii* is in agreement with previous MLST analysis (Inoue *et al.* 2009), and may be related to different migration patterns of the hosts. All *B. grahamii* isolates analysed in this study were taken from wild mice and voles, which migrate slowly and typically have a home area of less than 1 ha (Corbet & Harris 1991). In contrast, most of the previously analysed *B. henselae* isolates were derived from domestic cats or humans, which travel much longer distances. Although a geographic pattern is also evident among different cat breeds (Lipinski *et al.* 2008), the presence of the same breeds in different parts of the world has obscured traces of their natural migration patterns.

The near-identity of all *B. quintana* strains is presumably caused by its recent emergence in the human population. Likewise, a possible explanation to the lower sequence divergence in *B. henselae* as compared with *B. grahamii* is that the *B. henselae* population emerged more recently. A much larger population size

and population turnover for rodents compared with cats will lead to a larger population size of *B. grahamii*, a higher accumulation of mutations per unit time and thereby a higher sequence diversity. It is however also possible that domestic cats only harbour a fraction of all diversity within *B. henselae*, and it would be interesting to determine whether strains from wild felines have the same relatively low levels of sequence divergence.

Another remarkable finding was that recombination frequencies appear to differ between *Bartonella* populations. We observed well-supported conflicts across trees for individual loci in *B. grahamii*, but not in *B. henselae*, in agreement with an estimated recombination over mutation ratio in the higher range ($r/m = 1.7$) for *B. grahamii* as compared with $r/m = 0.1$ for *B. henselae* (Vos & Didelot 2009). This difference may be due to the higher density of wild rodents in nature, more frequent co-infections of different *Bartonella* species, and a higher exposure to vectors than for domestic cats. Typically, intracellular bacteria have low rates of recombination and horizontal gene acquisition (Vos & Didelot 2009), probably because of their isolated growth niche, lack of mobile elements and limited exposure to foreign DNA (Moran & Plague 2004). A notable exception is the A-group strains of *Wolbachia pipientis*, where it is estimated that up to 75% of all genes might have undergone recent recombination events, possibly mediated by transfer of DNA via bacteriophages across strains that infect the same insect host (Klasson *et al.* 2009). The high recombination frequencies in *B. grahamii* may be explained by the prevalence of plasmids in this species (Seubert *et al.* 2003a; Berglund *et al.* 2009), or the recently described GTA that packages random bacterial DNA (Berglund *et al.* 2009). With the aid of the GTA, large-scale gene transfer and sequence exchange is possible not only between strains that infect the same host or vector simultaneously but also between strains infecting at different time points.

Also in terms of gene content and genome size, *B. grahamii* displays more variation than what was previously observed in *B. henselae* (Lindroos *et al.* 2006). Still, homologous genes are variable in both the *B. grahamii* and the *B. henselae* populations, including genes for T4SSs, T5SSs, membrane proteins and phage genes. The genome sizes of several *B. grahamii* strains were estimated to be larger than that of the sequenced strain, suggesting that additional genes are present in the *B. grahamii* pan-genome. The identification of phage gene remnants in several sequenced spacer regions further indicates a continuous flux in the mobile gene pool of *B. grahamii*. A higher exposure to bacteriophages in the rodent-associated lineages may also contribute to elevated recombination frequencies.

Geographic variations in the genomic diversity: age, recombination and/or recent bottlenecks?

Our results revealed a striking difference in sequence diversity between Asian and European-American *B. grahamii* strains, with the Asian strains being much more divergent and showing more extensive recombination. As in the comparison of diversity among species, we can think of several possible reasons for this: the Asian population is older, larger and/or subjected to increased recombination frequencies. An alternative explanation is that the American–European population has undergone a recent bottleneck.

This raises interesting questions about the age of *Bartonella* infections in rodents, where the ancestor of the *B. grahamii* population originated, and how it has migrated since then. In the previous study by Inoue *et al.* (2009) it was suggested that *B. grahamii* existed in North America before Europe, and crossed the Bering's strait during a glacial epoch to the Euroasian continent. This hypothesis was based on the *gltA* sequence from a *B. grahamii* isolate from a *Mus musculus* in California, which appeared to be the earliest diverging lineage in the European–American group. When we repeated this analysis, including additional sequences, we found that the American strain Mo12658sd and a strain from Greece appear to represent the earliest diverging lineage in the European–American group, suggesting that we cannot say whether the origin of this group was in Europe or America. However, it is important to recall that the limited sequence information contained within the *gltA* gene is not enough to infer reliable phylogenies, that the diversity that we know today is probably only a fraction of what is out there, and that the earliest diverging lineage may not be there anymore.

Although it is tempting to speculate that the continent with the highest diversity represents the source of a population, we have conclusively shown that the higher genetic diversity in Asia does not by necessity imply that the ancestor was derived from this continent. If so, we would expect the American–European strains to represent an expansion from within the Asian clade. Rather, we observed two well-supported clades, suggesting that the origin could be at either side of the Bering's strait. Hence, we have no evidence to say that one population is older than the other.

Higher recombination frequencies would be a simple explanation for the observed higher diversity in the Asian population. Recombination frequencies across strains are likely to increase with the opportunities for multiple infections of the same host individual, which in turn will depend on the extent of host-specificity and the density of hosts and vectors. The overall prevalence of *Bartonella* in the rodent populations we have studied ran-

ged from 17% in Sweden (Holmberg *et al.* 2003) to 64% in Russia (Markov *et al.* 2006). However, large variations in abundance have been observed within countries, for example, in Japan where the *Bartonella* prevalence was over 80% in two of the geographic regions (Nagano and Ehime) from which the isolates in this study were sampled (Inoue *et al.* 2008). Furthermore, co-infections of different species within a single host have been demonstrated in Japan (Inoue *et al.* 2008), suggesting that these strains have indeed had opportunities for recombination. However, we cannot exclude that recombination occurs with similarly high frequencies also within the European and American populations, but has escaped detection in this analysis because of high sequence similarity across strains and limited sampling.

Another possible reason to why American and European populations are less diverse is that they can have experienced bottlenecks associated with the latest glacial period, which ended approximately 10 000 years ago (Ehlers & Gibbard 2007). Indeed, most of the samples from these two continents are derived from sites covered by ice at the glacial maximum, *c.* 18 000 years ago (Ehlers & Gibbard 2007) (Fig. S9, Supporting Information). Numerous studies of genetic diversity among rodents in Europe and North America have identified areas that were used as refuges during this time, and shown that genetic diversity decreases with distance from these refuges (Lessa *et al.* 2003; Michaux *et al.* 2003; Rowe *et al.* 2004; Kotlik *et al.* 2006). If only a limited number of rodents made it back to the previously ice-covered areas, then this is likely to have reduced the variability also of their blood-borne inhabitants. Interestingly, our local populations in UK and Canada display approximately the same degree of diversity as has been observed for human pathogens inferred to have emerged within the past 20 000 years (Achtman 2004, 2008), while the diversity of *B. grahamii* in the entire European–American group is much greater (Table S5, Supporting Information). Thus, if diversity in these regions was reduced as an effect of the ice age, it seems likely that rodents infected with genetically different *Bartonella* strains initiated the re-colonization.

Other factors influencing the infection dynamics such as population size variation, climate differences and seasonal sampling time may also contribute to the observed sequence divergence variation across continents. More ecological data and an ecological model are clearly needed to identify the selective and ecological factors underlying the apparent high recombination frequencies among *B. grahamii* strains in Asia. In the future, systematic surveys of the genomic diversity of rodent-associated bacterial populations along with collections of demographic data on the abundance, migration and diversity of rodents and their vectors will be invaluable for elucidating the historical and ecological factors that influence the genomic diversity of zoonotic bacterial species.

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Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Phylogenetic tree inferred from the concatenation of all gene sequences. Branch lengths are according to maximum likelihood, and bootstrap support values for the major clades are shown. The branch length of *B. vinsonii* is not according to scale.

Fig. S2 Phylogenetic tree inferred from the concatenation of all sequenced loci after removing all sites with gaps. Branch lengths are according to maximum likelihood, and bootstrap support values for the major clades are shown. The branch length of *B. vinsonii* is not according to scale.

Fig. S3 Phylogenetic tree based on partial *gltA* sequences (269 bp) of the *B. grahamii* strains analyzed in this study, and additional sequences from other parts of the world. Branch lengths are according to maximum likelihood, and bootstrap support values for the major clades are shown. Origin and accession numbers are shown for genes not sequenced as part of this study.

Fig. S4 Phylogenetic tree for locus 5. Branch lengths are according to maximum likelihood, and bootstrap support values over 75 are shown. The branch length of *B. vinsonii* is not according to scale.

Fig. S5 Consensus tree from ClonalFrame. Posterior probabilities for the major clades are shown.

Fig. S6 Schematic representation of PFGE results with the NotI enzyme for all strains (left). Since the size of the largest NotI band is difficult to estimate accurately, we also applied another enzyme, AscI, to a selected set of strains (right). The country of origin and estimated genome size for each strain are shown. The large difference in estimated genome size of strain Ehime 5-1 is probably due to an undetected band or a double band in the NotI digest.

Fig. S7 Circular representation of the microarray results for the plasmid pBGR3. The innermost circle shows the plasmid genes (magenta indicates the *vbh* genes). The strains are: 1, V2; 2, WM11; 3, PTZA 30/3; 4, PTZB 29/18; 5, J142; 6, C066; 7, R170; 8, J019; 9, S116; 10, MAC29; 11, C162; 12, Cg4228alb; 13, Cg4224alb; 14, Cg4227alb; 15, Cg4263alb; 16, Cg4285alb; 17, Mo12494sd; 18, Mo12658sd; 19, Ac1733yn; 20, AL1714yn; 21, Ehime 5-1; 22, Nakanoshima 39-1; 23, Nagano 14-1; 24, Fuji 4-1; 25, Hokkaido 29-1.

Fig. S8 Hybridization results of the *fha*-repeat in BgGI 4 (top, two of the three *fha/hec* operons in this island are shown), BgGI 14 (bottom, left) and BgGI 7 (bottom, right). In each image, the panels show from the top: the frequency of repeats in the *B. grahamii* as4aup genome (the number at which each sequence occurs in the genome with more than 80% sequence identity over 100 bp); the *B. grahamii* as4aup genes on the two strands with phage genes shown in orange; hybridization results for each strain. Rhombi below a gene indicate an integrase gene or remnant.

Fig. S9 Map showing the approximate extension of the latest glacial epoch. The sites where the bacterial isolates included in this study were sampled are indicated (only one site is shown for each country). Within parenthesis is the number of strains from each country.

Table S1 EMBL accession numbers for the eleven sequenced loci

Table S2 Sequence similarities within and between phylogenetic or geographic groups

Table S3 Predicted recombination events within sequenced loci

Table S4 Predicted recombination events in the MLST dataset from (Inoue *et al.* 2009)

Table S5 Sequence divergence and estimated divergence time of genetically monomorphic pathogens and our *B. grahamii* populations

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Bartonella japonica sp. nov. and *Bartonella silvatica* sp. nov., isolated from *Apodemus* mice

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Two bacterial strains, Fuji 18-1^T and Fuji 23-1^T, were isolated from the blood of the small Japanese field mouse (*Apodemus argenteus*) and the large Japanese field mouse (*Apodemus speciosus*), respectively, specimens of which were captured in the forest of Mount Fuji, Japan. Phenotypic characterization (growth conditions, incubation periods, biochemical properties and cell morphologies), DNA G+C contents (40.1 mol% for strain Fuji 18-1^T and 40.4 mol% for strain Fuji 23-1^T) and sequence analyses of the 16S rRNA genes indicated that both strains were members of the genus *Bartonella*. Using *rpoB* and *gltA* sequencing analysis, the highest sequence similarities between strains Fuji 18-1^T, Fuji 23-1^T and other recognized species of the genus *Bartonella* showed values considerably lower than 91.4 % and 89.9 % in the *rpoB* gene and 89.1 % and 90.4 % in the *gltA* gene, respectively. It is known that similarities of 95.4 % for the *rpoB* gene and 96.0 % for the *gltA* gene can be applied as cut-off values for the designation of novel species of the genus *Bartonella*. In a phylogenetic tree based on the merged set of concatenated sequences of seven loci [16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and the intergenic spacer region (ITS)], strains Fuji 18-1^T and Fuji 23-1^T formed a distinct clade from other recognized species of the genus *Bartonella*. These data support the classification of strains Fuji 18-1^T and Fuji 23-1^T as novel species of the genus *Bartonella*. The names *Bartonella japonica* sp. nov. and *Bartonella silvatica* sp. nov. are proposed for these novel species. The type strains of *Bartonella japonica* sp. nov. and *Bartonella silvatica* sp. nov. are Fuji 18-1^T (=JCM 15567^T=CIP 109861^T) and Fuji 23-1^T (=JCM 15566^T=CIP 109862^T), respectively.

Abbreviations: ITS, intergenic spacer region; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank accession numbers for the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and the 16S–23S rRNA intergenic spacer region (ITS) from *Bartonella japonica* sp. nov. Fuji 18-1^T are AB440632, AB440633, AB242289, AB440634, AB440635, AB242288, and AB498007, respectively. GenBank accession numbers for the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and ITS sequences from *Bartonella silvatica* sp. nov. Fuji 23-1^T are AB440636, AB440637, AB242287, AB440638, AB440639, AB242292, and AB498008, respectively.

Sequence similarities of the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and ITS region sequences between strains Fuji 18-1^T, Fuji 23-1^T and other species of the genus *Bartonella*, a maximum-parsimony phylogenetic tree based on the concatenated sequence of the seven loci and neighbour-joining trees of the seven loci are available as supplementary material with the online version of this paper.

The genus *Bartonella* is classified in the class *Alphaproteobacteria*, order *Rhizobiales* and family *Bartonellaceae* and, at the time of writing, comprised 19 recognized species and three subspecies. The bacteria in the genus *Bartonella* are small, fastidious, slow-growing Gram-negative rods (Brenner *et al.*, 1993; Birtles *et al.*, 1995). Species of the genus *Bartonella* are known to infect erythrocytes and endothelial cells of various mammals, such as humans, cats, dogs, ruminants, wild rabbits and wild rodents (Dehio, 2005). We previously described the prevalence of species of the genus *Bartonella* among 685 wild rodents in Japan (Inoue *et al.*, 2008). Based on phylogenetic trees constructed with the *rpoB* and *gltA* gene sequences and the criteria for the definition of a species of the genus *Bartonella* (La Scola *et al.*, 2003), two strains were identified as possible novel species of this genus. Strains Fuji 18-1^T and Fuji 23-1^T were isolated from *Apodemus argenteus* and *Apodemus speciosus* mice captured in the Mount Fuji forest in Japan. In the present study, these strains were characterized by biochemical, morphological and genetic approaches, including multilocus sequencing analysis of six housekeeping genes and the 16S–23S rRNA intergenic spacer region (ITS).

Strains Fuji 18-1^T and Fuji 23-1^T were grown on heart infusion agar plates (HIA; Difco) containing 5% (w/v) defibrinated rabbit blood at 35 °C with 5% CO_2 for 14 days. Gram staining was assessed by light microscopy (Olympus) at × 1000 magnification. Cell morphology was observed by transmission electron microscopy (model JEM1200EX; JEOL) at 100 kV with negative staining.

Biochemical characteristics were assessed by using a MicroScan Rapid Anaerobe Panel (Dade Behring Inc.) according to the manufacturer's instructions as described previously (Welch *et al.*, 1993). Cytochrome oxidase test strips (Nissui) were used for evaluating the oxidase activity of the strains. Catalase activity was examined by mixing fresh colonies which had been cultured for 14 days at 35 °C on 5% rabbit blood chocolate HIA plates with 3% H_2O_2 on a glass slide.

DNA G+C content was determined by HPLC (Mesbah & Whitman, 1989). Mean values of the G+C content (±SD) were calculated based on assays conducted in triplicate.

Genomic DNA was extracted using the Instagene Matrix (Bio-Rad) according to the manufacturer's instructions. Six housekeeping genes, 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB*, and ITS fragments were amplified by PCR as previously described (Birtles & Raoult, 1996; Heller *et al.*, 1997; Renesto *et al.*, 2001; Houpikian & Raoult, 2001; Zeaiter *et al.*, 2002a, b; Inoue *et al.*, 2009). The PCR products were purified using a Spin Column PCR Product Purification kit (Bio Basic). Direct DNA sequencing of the purified PCR products was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) on a Genetic Analyzer (model 3130; Applied Biosystems). For the phylogenetic analysis, sequence data were aligned with those of type strains of other species of the genus *Bartonella* (Table 1) that were available in GenBank by using CLUSTAL W software (Thompson *et al.*, 1994) in the MEGA4 program (Tamura *et al.*, 2007). Phylogenetic trees based on six housekeeping genes and the ITS region were constructed using the neighbour-joining (NJ) method (Saitou & Nei, 1987). The nucleotide substitution rates were calculated by Kimura's two-parameter distance model (Kimura, 1980). Bootstrap analysis was carried out on 1000 replications of the dataset (Felsenstein, 1985). *Brucella melitensis* 16M^T was chosen as the outgroup. The phylogenetic trees of the concatenated sequence data for the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and the ITS region were constructed using the NJ and maximum-parsimony (MP) methods (Fitch, 1971) with the Jukes–Cantor parameter model (Jukes & Cantor, 1969) by using the MEGA4 program.

Strains Fuji 18-1^T and Fuji 23-1^T grown on HIA formed smooth, transparent to grey-whitish colonies of 1–2 mm in diameter. Gram-negative coccobacilli to short rod-shaped cells were observed by light microscopy after 14 days culture. The cell morphologies of both strains were similar and no flagella or pili were observed by electron microscopy (Fig. 1). Cell sizes were 0.74 μm in length and 0.36 μm in width for strain Fuji 18-1^T and 1.16 μm in length and 0.43 μm in width for strain Fuji 23-1^T.

Both strains were oxidase- and catalase-negative and neither exhibited urease activity nor hydrolysed trehalose. They both hydrolysed bis-*p*-nitrophenyl phosphate, but not *p*-nitrophenyl *N*-acetyl β-D-glucosaminide. Both strains had amino acid arylamidase activity towards leucine, methionine, lysine (alkaline as well as acidic), glycine, arginine and tryptophan. Strain Fuji 23-1^T exhibited L-proline-β-naphthylamide activity but strain Fuji 18-1^T did not. Both strains had glycylglycylarylamidase activity, but not pyrrolidonyl arylamidase activity. These biochemical results are typical for members of the genus *Bartonella* (Bermond *et al.*, 2000, 2002; Maillard *et al.*, 2004); however, the profiles cannot be applied routinely and reliably for the differentiation of species of the genus *Bartonella* because of the relatively inert nature of bartonellae (Dehio *et al.*, 2001; Bermond *et al.*, 2002).

The DNA G+C contents of strains Fuji 18-1^T and Fuji 23-1^T were 40.1±0.6 mol% (mean±SD) and 40.4±0.5 mol% (mean±SD), respectively. These values were similar to those of other species of the genus *Bartonella* (Bermond *et al.*, 2000, 2002).

DNA fragments of all seven loci examined were sequenced and the sequence data were compared with the type strains of other species of the genus *Bartonella*. The 16S rRNA gene sequence similarities of strains Fuji 18-1^T and Fuji 23-1^T to other *Bartonella* species ranged from 96.5% (*Bartonella bacilliformis*) to 98.8% (*Bartonella grahamii*) and 96.7% (*Bartonella bacilliformis*) to 98.8% (*Bartonella koehlerae*), respectively (see Supplementary Table S1 in IJSEM Online).

Table 1. GenBank accession numbers of the seven loci for the *Bartonella* species used in this study

Species	GenBank accession numbers						
	16S rRNA	*ftsZ*	*gltA*	*groEL*	*ribC*	*rpoB*	ITS
Bartonella japonica sp. nov. Fuji 18-1^T (=JCM 15567^T=CIP 109861^T)	AB440632	AB440633	AB242289	AB440634	AB242288	AB242288	AB498007
Bartonella silvatica sp. nov. Fuji 23-1^T (=JCM 15566^T=CIP 109862^T)	AB440636	AB440637	AB242287	AB440638	AB440639	AB242292	AB498008
B. alsatica IBS 382^T (=CIP 105477^T)	AJ002139	AF467763	AF204273	AF299357	AY116630	AF165987	AF312506
B. bacilliformis KC583^T (=ATCC 35685^T)	Z11683	AB292602	AB292601	Z11683	AJ236918	AF165988	CP000524
B. birtlesii IBS 325^T (=CIP 106294^T)	AF204274	AF467762	AF204272	AF355773	AY116632	AB196425	AY116640
B. bovis 91-4^T (=CIP 106692^T)	AF293391	AF467761	AF293394	AF071194	AY116637	AY166581	AY116638
B. capreoli IBS 193^T (=CIP 106691^T)	AF293389	AB290192	AF293392	AB290190	AB290194	AB290188	AB498009
B. chomelii A828^T (=CIP 107869^T)	AY254309	AB290193	AY254308	AB290191	AB290195	AB290189	AB498010
B. clarridgeiae Houston-2 cat^T (=ATCC 51734^T)	AB292603	AF141018	U84386	AF014831	AB292604	AF165990	AF312497
B. doshiae R18^T (=NCTC 12862^T)	Z31351	AF467754	Z70017	AF014832	AY116627	AF165991	AJ269786
B. elizabethae F9251^T (=ATCC 49927^T)	L01260	AF467760	Z70009	AF014834	AY116633	AF165992	L35103
B. grahamii V2^T (=NCTC 12860^T)	Z31349	AF467753	Z70016	AF014833	AY166583	AF165993	AJ269785
B. henselae Houston-1^T (=ATCC 49882^T)	BX897699	AF061746	BX897699	AF014829	AJ132928	AF171070	L35101
B. koehlerae C-29^T (=ATCC 700693^T)	AF076237	AF467755	AF176091	AY116641	AY116634	AY166580	AF312490
B. quintana Fuller^T (=ATCC VR-358^T)	M11927	AB292605	Z70014	AB290196	AJ236917	AF165994	L35100
B. schoenbuchensis R1^T (=NCTC 13165^T)	AJ278187	AF467765	AJ278183	AY116642	AY116628	AY167409	AY116639
B. taylorii M6^T (=CIP 107028^T)	Z31350	AF467756	Z70013	AF304017	AY116635	AF165995	AJ269788
B. tribocorum IBS 506^T (=CIP 105476^T)	AJ003070	AF467759	AJ005494	AF304018	AB292600	AF165996	AF312505
B. vinsonii subsp. *arupensis* OK 94-513^T (=ATCC 700727^T)	AF214558	AF467758	AF214557	AF304016	AY116631	AY166582	AF312504
B. vinsonii subsp. *berkhoffii* 93-CO1^T (=ATCC 51672^T)	L35052	AF467764	U28075	AF014836	AY116629	AF165989	AF167988
B. vinsonii subsp. *vinsonii* Baker^T (=ATCC VR-152^T)	M73230	AF467757	Z70015	AF014835	AY116636	AF165997	L35102

La Scola *et al.* (2003) reported that *rpoB* and *gltA* were the most appropriate genes for discriminating species of the genus *Bartonella* and proposed that gene sequence similarities <95.4 % in *rpoB* and <96.0 % in *gltA* between recognized species of the genus *Bartonella* could be used as cut-off values for the designation of novel *Bartonella* species. The highest sequence similarities between strains Fuji 18-1^T, Fuji 23-1^T and other species of the genus *Bartonella* showed values considerably lower than 91.4 % (strain Fuji 18-1^T compared with *Bartonella alsatica*) and 89.9 % (strain Fuji 23-1^T/*B. alsatica*) for the *rpoB* gene and 89.1 % (strain Fuji 18-1^T/*Bartonella vinsonii* subsp. *arupensis*) and 90.4 % (strain Fuji 23-1^T/*Bartonella taylorii*) for the *gltA* gene. Thus, strains Fuji 18-1^T and Fuji 23-1^T fulfil the requirements for classification as novel species of the genus *Bartonella*.

Strains Fuji 18-1^T and Fuji 23-1^T also showed considerably lower gene sequence similarities for the remaining four loci, *ftsZ*, *groEL*, *ribC* and ITS, when compared with other recognized species of the genus *Bartonella* (Supplementary Table S1). In the phylogenetic tree based on the merged set of concatenated sequences of seven loci, strains Fuji 18-1^T and Fuji 23-1^T formed a distinct clade with other species of the genus *Bartonella* (Fig. 2). The MP tree based on the concatenated sequence of the seven loci and the NJ trees based on sequence analyses of the seven loci also revealed that strains Fuji 18-1^T and Fuji 23-1^T were clearly separated from all other species of the genus *Bartonella* (see Supplementary Figs S1 and S2 in IJSEM Online).

In conclusion, sequence similarities of the *rpoB* and *gltA* genes and the phylogenetic analyses of seven different loci support the classification of strains Fuji 18-1^T and Fuji 23-1^T as novel species of the genus *Bartonella*, for which we propose the names *Bartonella japonica* sp. nov. and *Bartonella silvatica* sp. nov., respectively.

Description of *Bartonella japonica* sp. nov.

Bartonella japonica [ja.po'ni.ca. N.L. fem. adj. *japonica* of Japan, where the host rodent, the small Japanese field mouse (*Apodemus argenteus*), from which the strain was isolated, is widely distributed].

After 14 days incubation on HIA at 35 °C in a moist atmosphere under 5 % CO_2, colonies appear small (1–2 mm in diameter), round, grey-whitish and smooth. Cells are small bacilli without flagella or pili and are 0.74 × 0.36 μm. Oxidase- and catalase-negative, does not exhibit urease activity or hydrolyse trehalose. Hydrolyses bis-*p*-nitrophenyl phosphate but not *p*-nitrophenyl *N*-acetyl β-D-glucosaminide. Exhibits arylamidase activity

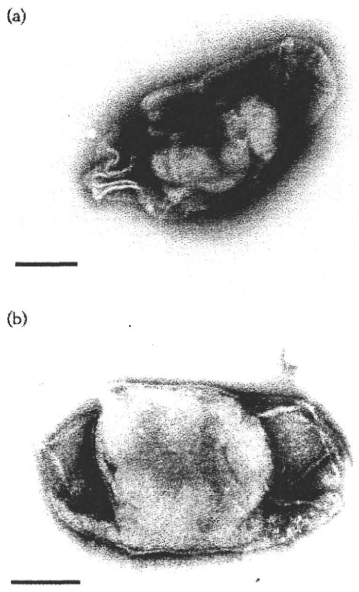

Fig. 1. Transmission electron micrograph of cells of (a) *Bartonella japonica* sp. nov. strain Fuji 18-1^T and (b) *Bartonella silvatica* sp. nov. strain Fuji 23-1^T. Bars, 200 nm.

towards leucine, methionine, lysine (alkaline as well as acidic), glycine, arginine and tryptophan, but not to proline. Glycylglycylarylamidase activity is present, but no pyrrolidonyl arylamidase activity. Can be distinguished from other species of the genus *Bartonella* by the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* gene and ITS region sequences.

The type strain, Fuji 18-1^T (=JCM 15567^T=CIP 109861^T), was isolated from the blood of *Apodemus argenteus* mice. The DNA G+C content of the type strain is 40.1 mol%.

Description of *Bartonella silvatica* sp. nov.

Bartonella silvatica [sil.va'ti.ca. L. fem. adj. *silvatica* of the forest where the host rodent, the large Japanese field mouse (*Apodemus speciosus*), from which the strain was isolated, was captured].

After 14 days incubation on HIA at 35 °C in a moist atmosphere under 5 % CO_2, colonies appear small (1–2 mm in diameter), round, grey-whitish and smooth. Cells are small bacilli without flagella or pili and are 1.16 × 0.43 μm. Oxidase- and catalase-negative. Does not exhibit urease activity or hydrolyse trehalose. Hydrolyses bis-*p*-nitrophenyl phosphate but not *p*-nitrophenyl *N*-acetyl β-D-glucosaminide. Exhibits arylamidase activity towards leucine, methionine, lysine (alkaline as well as acidic), glycine, proline, arginine and tryptophan. Glycylglycylarylamidase activity, but no pyrrolidonyl arylamidase activity. Can be distinguished from other species of the genus *Bartonella* by the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* gene and ITS region sequences.

The type strain, Fuji 23-1^T (=JCM 15566^T=CIP 109862^T), was isolated from the blood of *Apodemus speciosus* mice. The DNA G+C content of the type strain is 40.4 mol%.

Acknowledgements

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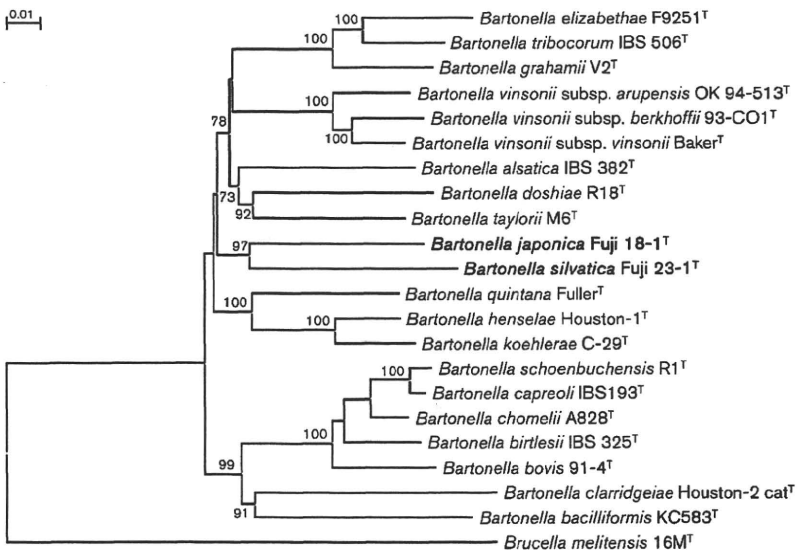

Fig. 2. Phylogenetic relationship of species of the genus *Bartonella* inferred from concatenated sequences of 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and the ITS region. The phylogenetic tree was constructed by using the NJ method with the Jukes–Cantor parameter model. The tree was rooted by the use of *Brucella melitensis* 16M^T as an outgroup. Bootstrap values (percentages of 1000 replications) above 70 % are indicated at the nodes. Bar, 0.01 estimated nucleotide substitutions per site.

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ORIGINAL ARTICLES

Detection of *Bartonella tamiae* DNA in Ectoparasites from Rodents in Thailand and Their Sequence Similarity with Bacterial Cultures from Thai Patients

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Abstract

Ectoparasites, including chigger mites (genera *Leptotrombidium, Schoengastia,* and *Blankarrtia*) and one tick (genus *Haemaphysalis*) collected from wild-caught rodents in Thailand, were assessed for the presence of *Bartonella* DNA by using a polymerase chain reaction assay targeting the 16S–23S intergenic spacer region and citrate synthase gene (*gltA*). Of the 41 pooled samples tested, 34 were positive for *Bartonella* DNA. Sequence analysis demonstrated that DNA detected in 33 chigger mite pools and one tick pool was similar to *Bartonella tamiae* sequences previously isolated from three patients in Thailand. This is the first report of the detection of *B. tamiae* DNA in chigger mites; additional field and experimental investigations are required to determine the role of chigger mites as potential vectors of *B. tamiae.*

Key Words: *Bartonella tamiae*—Chigger mite—Ectoparasites—Rodent—Thailand—Tick.

Introduction

Organisms of the genus *Bartonella* are aerobic, fastidious, gram-negative, and slow-growing bacteria, many of which have been isolated from the blood of mammals. This genus currently consists of at least 20 named species and 3 subspecies (Chomel et al. 2006); of these, at least 13 species have been reported to be potential causative agents for a wide spectrum of human diseases (Chomel et al. 2009). A variety of *Bartonella* species have been isolated from wild rodents, four of which were suspected to have caused clinical manifestations in humans (Daly et al. 1993, Kerkhoff et al. 1999, Welch et al. 1999, Kosoy et al. 2003, Fenollar et al. 2005). Previous reports indicating a high prevalence of *Bartonella* infection in wild rodents makes these bacteria a potential public health concern (Inoue et al. 2008).

Bloodsucking arthropods have been shown to be involved in the transmission of *Bartonella* species among their hosts (Billeter et al. 2008a). Sandflies (*Lutzomyia* spp.), the human body louse (*Pediculus humanus humanus*), and the cat flea (*Ctenocephalides felis*) have been implicated in the transmission of *B. bacilliformis, B. quintana,* and *B. henselae,* respectively (Chomel et al. 1996, Raoult and Roux 1999, Karem et al. 2000). Ticks were suspected to be involved in the transmissions of *B. vinsonii* subsp. *berkhoffii* and *B. vinsonii* subsp. *arupensis* (Pappalardo et al. 1997, Welch et al. 1999, Chang et al. 2001), and recent experimental study suggests that *Ixodes ricinus* may be involved in the transmission of *B. henselae* (Cotté et al. 2008). The DNA of *Bartonella* species has been detected in fleas collected from diverse wild rodents (Stevenson et al. 2003, Marié et al. 2006, Morway et al. 2008). In addition, *Bartonella* DNA was found in keds, biting flies, mites, and miscellaneous arthropods (Billeter et al. 2008a). These results demonstrated the potential role of these various insects as vectors for the transmission of *Bartonella* sp. among mammalian hosts.

Kosoy and colleagues (2008) recently isolated a novel *Bartonella* sp., *B. tamiae,* from three patients in Thailand. The patients showed symptoms typical for bartonellosis, such as fever, mild anemia, and ocular disorder. Although these patients had reported contact with rats, the source and/or possible vectors of the infection remains unknown. Recent detection of *B. tamiae*-like DNA in *Amblyomma americanum*

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ticks (Acari: Ixodidae) collected in Virginia also suggests a potential role of ticks in the transmission of *B. tamiae* (Billeter et al. 2008).

More than 250 species of mites are associated with health-related problems for humans and domestic animals (Mullen and Durden 2002). Probably foremost among these problems are temporary irritation of the skin, dermatitis, and allergies. There are very few human diseases caused by pathogens transmitted by mites, with two significant exceptions: rickettsialpox and scrub typhus (Mullen and Durden 2002). Several studies have implicated mites of the superfamily Dermanyssoidea in the transmission of bacterial pathogens, such as *Salmonella*, *Pasteurella*, and *Borrelia* (Netusil et al. 2005, Valiente et al. 2005). *Orientia tsutsugamushi*, the etiological agent of scrub typhus, is the only known pathogen transmitted by trombiculid mites (genus *Trombicula*) (Lerdthusnee et al. 2003).

The aim of this study was to evaluate mites and other ectoparasites for the presence of *Bartonella*-specific DNA.

Materials and Methods

Ectoparasites were collected from 41 rodents of five species: *Rattus rattus* (29), *Rattus argentiventer* (2), *Bandicota indica* (5), *Bandicota savilei* (3), and *Mus cervicolor* (2). These rodents were captured from five different regions of Thailand: the northern region (Nam province, $n = 7$), the northeastern region (Ubon Ratchathani and Ubon Thani provinces, $n = 8$), the eastern region (Chon Buri province, $n = 1$), the central region (Nonthaburi and Pha Nakon Si Ayutthaya provinces, $n = 3$), and the southern region (Nakhon Si Thammarat, Surat Thani, and Yala provinces, $n = 22$).

The ectoparasites were morphologically identified to genus. The 209 collected mites belonged to the genera *Leptotrombidium* (130), *Schoengastia* (67), and *Blankarrtia* (12), and eight collected ticks belonged to the genus *Haemaphysalis*. The mites collected from each animal were pooled for testing by mite genus. The number of mites in each pool was from 1 to 9 (mean, 5.3). The eight ticks were collected from one mouse and were also pooled together. In total, we tested 40 pools of 209 mites and one pool of eight ticks. The 40 tested mite pools belonged to the genera *Leptotrombidium* (24), *Schoengastia* (14), and *Blankarrtia* (2). DNA was extracted using the Qiagen Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol for blood and body fluid, with minor modifications. Briefly, polymerase chain reaction (PCR) was performed in 25 μL of mixtures containing 12.5 μL of 2×iQ SYBR Green Supermix (BioRad, Hercules, CA), 0.5 pmol of each primer, and 2.5 μL of template DNA. *Bartonella* DNA was amplified using primers for intergenic spacer (ITS) (Billeter et al. 2008b) and for *gltA* specifically designed for detection of *B. tamiae* (BtGLT5′: TTC CTG AGT TTG TAG CAA AA; BtGLT3′: GGA TCA TCT TTA ATG CCC AA). The amplification of DNA was performed on a thermal cycler (iCycler; BioRad). Briefly, for ITS, 1 cycle for 3 min at 95°C was followed by 55 cycles for 30 s at 95°C, 30 s at 57°C, and 30 s at 72°C, and a final extension cycle for 7 min at 72°C; and for *gltA*, 1 cycle for 4 min at 94°C, followed by 45 cycles for 30 s at 95°C, 60 s at 49°C, and 30 s at 72°C, and a final extension cycle for 7 min at 72°C. Ten microliters of each PCR product was run on a 1.5% agarose gel (Agarose; Promega, Madison, WI).

Positive PCR products were sequenced using an Applied Biosystems Model 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The CLUSTAL-X program was used for the phylogenetic analysis of the obtained sequences. The neighbor-joining method by Kimura's two-parameter distance method and bootstrap calculation was carried out for 1000 resamplings. MEGA 4.0.1 software (The Biodesign Institute, Tempe, AZ) was used for these analyses.

Results

Of the 40 pooled mite samples, 29 (72.5%) and 9 (22.5%) were positive for *B. tamiae* based on ITS and *gltA*, respectively (Table 1). Among these, five samples were positive for both genes (12.5%). One tick sample was also positive for *B. tamiae* for ITS. The length of the PCR amplicons for ITS and *gltA* were 230–242 and 114–116 bp, respectively.

Sequence analysis of the ITS amplicons revealed two distinct sequence groups, one of which formed a cluster with *B. tamiae* strain Th239 (ITS239) and the other with strains Th307 and Th339 (ITS307/339), which were previously isolated from humans (Kosoy et al. 2008) (Fig. 1). Fourteen of the 30 ITS sequences (34.2% of tested) grouped with the ITS239 sequence, whereas the other 16 (39.0%) grouped with the ITS307/339 sequence (Table 2). These groups can be distinguished by the presence of 12-bp insertion in the amplified ITS region; this insertion was present only in ITS239 but not in ITS307/339 (data not shown). Analysis of the *gltA* sequences also revealed two distinct groups, and both groups were closer to strains Th239/307 than to Th339 (Fig. 2).

The sequence groups of ITS239 and ITS307/339 were 29.2% (7/24) and 37.5% (9/24), respectively, among mites of genus *Leptotrombidium*, 35.7% (5/14) and 42.9% (6/14) among mites of genus *Schoengastia*, and 100% (2/2) and 0% (0/2) among mites of genus *Blankarrtia*, respectively (Table 2). The two sequence groups were detected in 20.7% (6/29) and 44.8% (13/29) of mites collected from *R. rattus*, 50.0% (1/2) and 0% (0/2) of mites from *R. argentiventer*, 80.0% (4/5) and 20.0% (1/5) of mites from *B. indica*, 66.7% (2/3) and 33.3% (1/3) of mites *B. savilei*, and 50% (1/2) and 50% (1/2) of mites from *M. cervicolor*, respectively (Table 3). Of the 14 total sequence positives found in the ITS239 group, 4 were isolated from ectoparasites collected in the northern region of Thailand, whereas the other 10 came from the southern region of Thailand. Of the 16 total ITS sequence positives in the ITS307/339 group, 3 were found in the northern region of Thailand, 6 from the northeastern region, 2 from the central region, and 5 from the southern region (Table 4).

TABLE 1. DETECTION OF *BARTONELLA TAMIAE* DNA IN CHIGGER MITES AND TICKS COLLECTED FROM THAI RODENTS BY POLYMERASE CHAIN REACTION TARGETING OF INTERGENIC SPACER (ITS) AND *GLTA*

		ITS		
		Positive number (%)	*Negative number (%)*	*Total number (%)*
gltA	Positive	5 (12.2)	4 (9.8)	9 (22.0)
	Negative	25[a] (61.0)	7 (17.1)	32 (78.1)
	Total	30[a] (73.2)	11 (26.8)	41[a] (100)

[a]This includes a tick pool.

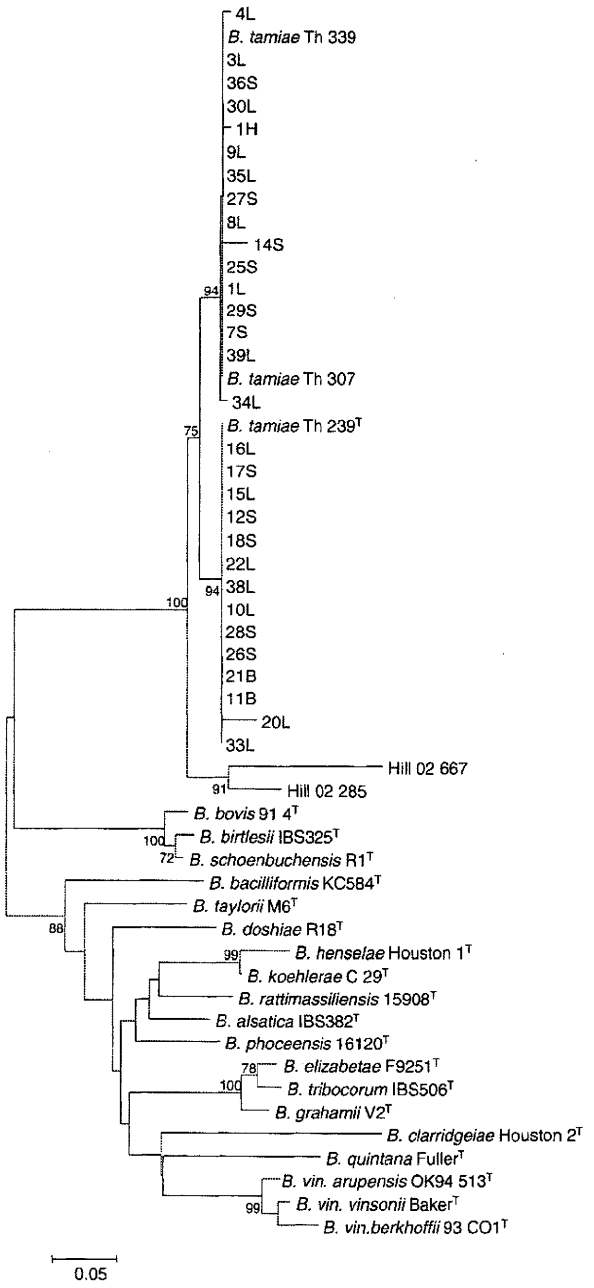

FIG. 1. Phylogenetic analysis of the sequences of intergenic spacer (ITS) from *Bartonella* detected in ectoparasites collected from Thai rodents. The phylogenetic tree was constructed by the neighbor-joining method; bootstrap values were estimated with 1000 replicates. Only bootstrap replicates >70% are noted. The 30 samples that were positive for ITS were classified into two (ITS239 and ITS307/339) clusters. The sequence detected from an *Amblyoma americanum* tick (Hill-02-28, Hill-02-66; Billeter et al. 2008b) was also included. Sample ID represents sample no., followed by the genus of ectoparasite (L for *Leptotrombidium*, S for *Schoengastia*, B for *Blankarrtia*, and H for *Haemaphysalis*).

TABLE 2. DETECTION OF *BARTONELLA* DNA INTERGENIC SPACER (ITS) FROM MITES AND TICKS INFESTING WILD RODENTS IN THAILAND: COMPARISON BY ECTOPARASITE

Ectoparasites	*Number tested*	*Number positive (%) for* Bartonella tamiae *sequence group*		
		I-239	*I-307/339*	*Total*
Leptotrombidium	24	7 (29.2)	9 (37.5)	16 (66.7)
Schoengastia	14	5 (35.7)	6 (42.9)	11 (8.6)
Blankarrtia	2	2 (100)	0 (0)	2 (100)
Haemaphysalis	1	0 (0)	1 (100)	1 (100)
Total	41	14 (34.1)	16 (39.0)	30 (73.2)

Discussion

In our study, we detected DNA specific for *B. tamiae* in mites collected from Thai rodents; this, therefore, represents one of the few reports showing a potential role of mites in the transmission of *Bartonella* (Durden et al. 2004, Billeter et al. 2008a). Percentage of the sequence homologies between the detected DNA in this study and those of the known *B. tamiae* were 97.8–100% for ITS307/339 group and 97.7–99.6% for ITS239 group, respectively, and 98.3–100% for the *gltA* gene. The first study on mites in the transmission of *Bartonella* species was reported more than 60 years ago when Baker (1946) showed that hamsters inoculated with suspension of mites collected from *Microtus* voles from Canada became bacteremic with an agent that was later identified as *B. vinsonii*. More recently, Kim et al. (2005) tested 21 Mesostigmatid mite pool samples and found 4 (19%) positive pools by PCR targeting the 16S rRNA gene; the DNA sequence showed a high homology (99.2%) with *B. doshiae*. Reeves et al. (2006) demonstrated that a *Steatonyssus* sp., a mite removed from a bat, harbored a *Bartonella* sp. (96%) that was closely related to an unnamed *Bartonella* found in rodents. Reeves et al. (2007) detected a part of the *groEL* gene from one of eight pools of tropical rat mites (*Ornithonyssus bacoti*) collected from *R. rattus* in Egypt by sequence analysis and demonstrated it to be an unique sequence with 81% similarity to a *Bartonella* species. In addition to mites associated with rodents, evidence of *Bartonella* DNA has been reported from mites of bats (Chiroptera, *Steatonyssus* sp.) and house dust mites (*Dermatophagoides farinae* and *D. pteronyssinus*) (Valerio et al. 2005, Reeves et al. 2006).

To the best of our knowledge, this is the first report of the presence of *Bartonella* DNA in the chigger mite. Durden et al. (2004) reported that none of the examined chigger mites collected from gray squirrels was positive for *Bartonella* by PCR targeting of *gltA* gene. Our study is also the first report showing the detection of *B. tamiae* DNA in chigger mites. *B. tamiae* is a novel *Bartonella* species that was isolated from patients in Thailand (Kosoy et al. 2008). Strains Th239, Th307, and Th339 were isolated from three separate patients. Although all the patients reported exposures to rats, the role of the rats in the transmission of *B. tamiae* has not been proved (Kosoy et al. 2008). Interestingly, the DNA sequence of *gltA* which we identified in mites infesting rodents were highly similar to those of *B. tamiae* isolated from Thai patients. These findings suggest a possible role of the chigger mites in the transmission of *B. tamiae* to humans.

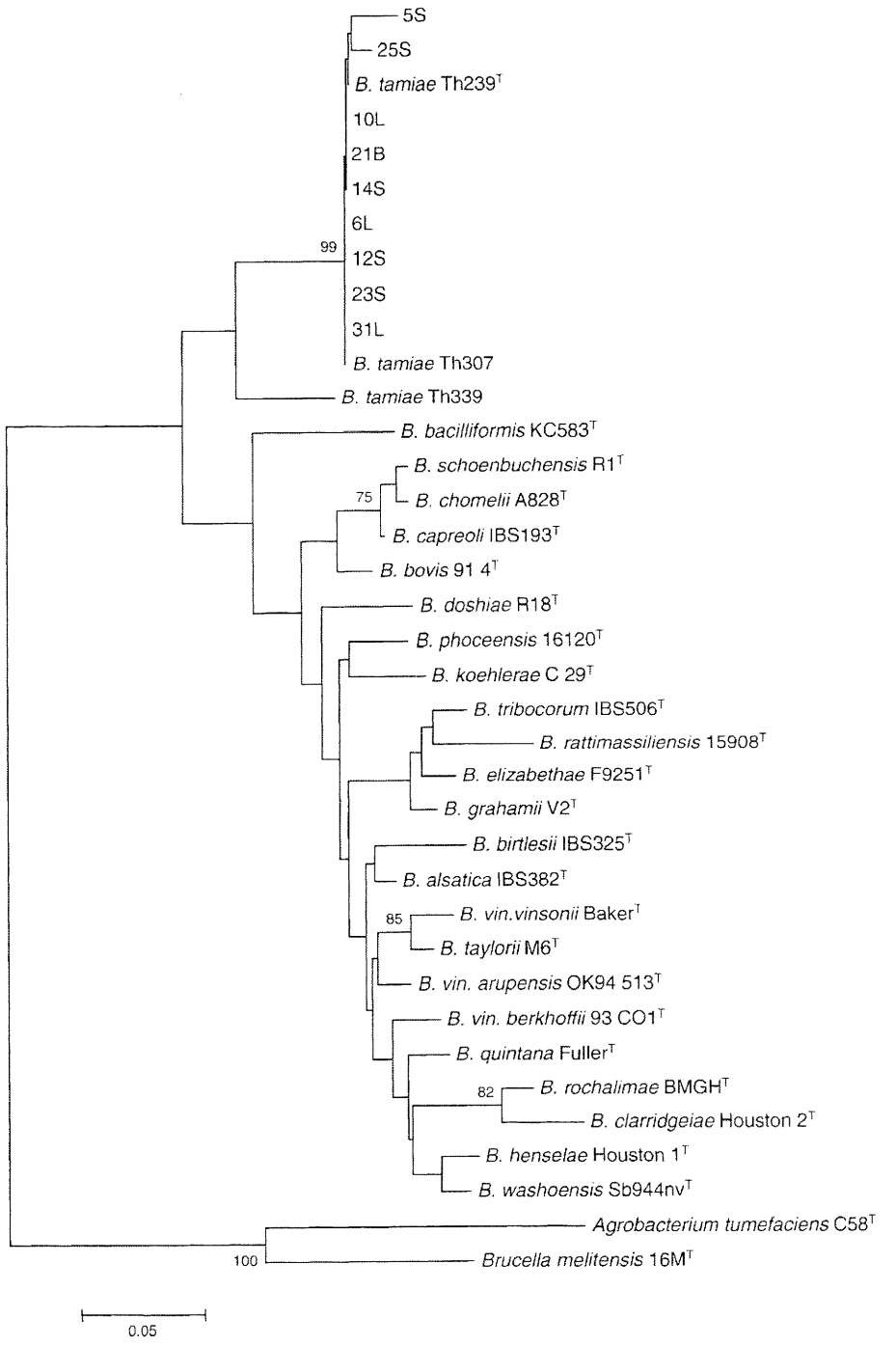

FIG. 2. Phylogenetic analysis of the sequences of *gltA* from *Bartonella* detected in ectoparasites collected from Thai rodents. The phylogenetic tree was constructed by the neighbor-joining method; bootstrap values were estimated with 1000 replicates. Only bootstrap replicates >70% are noted. The nine samples that were positive for *gltA* were classified into the same cluster. Sample ID represents sample no., followed by the genus of ectoparasite (L for *Leptotrombidium*, S for *Schoengastia*, and B for *Blankarrtia*). The sequences from *Brucella melitensis* 16 M^T and *Agrobacterium tumefaciens* $C58^T$ were used as out-group bacteria.

One tick of the genus *Haemaphysalis* was positive for DNA specific for the sequence group of ITS307/339. Recently, Billeter et al. (2008b) also reported the presence of *B. tamiae*-like sequences in two *A. americanum* by using the same primer pairs that were used in our study. Thus, ticks as well as chigger mites might play a role in the transmission of *B. tamiae*.

We identified two distinct sequence groups, ITS307/339 and ITS239, which show high sequence similarity with the isolates from the patients. The 16 sequences belonging to the