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## New World origins for haemoparasites infecting United Kingdom grey squirrels (*Sciurus carolinensis*), as revealed by phylogenetic analysis of bartonella infecting squirrel populations in England and the United States

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### SUMMARY

Phylogenetic analyses of bartonella have suggested divergence between bartonellae that infect mammals native to the Old and New Worlds. We characterized bartonella isolated from Eastern grey squirrels (*Sciurus carolinensis*) in the United States and from grey and red squirrels (*Sciurus vulgaris*) in the United Kingdom by nucleotide sequence comparison (*gltA* and *groEL*). Isolates from grey squirrels in the United States and the United Kingdom were identical, and most similar to *Bartonella vinsonii*, a species associated with New World rodents. A single and novel bartonella genotype was obtained from all 12 red squirrel isolates. Although grey squirrels were first introduced into the United Kingdom over 125 years ago, they continue to be infected solely by the bartonella associated with grey squirrels native to the United States. These results illustrate that exotic species may be accompanied by the introduction and maintenance, over many generations, of their microparasites.

### INTRODUCTION

The genus *Bartonella* consists of 13 described species of haemotrophic, Gram-negative bacteria, all of which have been isolated from mammals including humans, companion animals such as cats and dogs, and wild rodents and lagomorphs [1, 2]. In addition, a large number of partially characterized strains have been isolated from hosts such as cattle, deer and numerous species of rodents [3–6]. Although these findings confirm a wide diversity of hosts, they may well represent only a fraction of the true scope of members

in the genus. Polymerase chain reaction (PCR)-based studies have suggested many *Bartonella* species may resist isolation using current culture methods [7] and, historically, bartonella-like organisms have been observed in blood smears prepared from fish, amphibians and birds as well as mammals [8].

Historically only two *Bartonella* species have been associated with disease in humans, *Bartonella bacilliformis* causing bartonellosis in the South American Andes and *B. quintana* causing trench fever. Other species including *B. henselae*, *B. clarridgeiae*, *B. vinsonii* subspecies *berkhoffii* and *arupensis*, *B. elizabethae* and *B. grahamii* have recently been implicated in a range of syndromes (e.g. cat scratch disease, bacillary angiomatosis, endocarditis and ocular infections

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[1, 9, 10]). This has led to the increased recognition of the genus as emerging pathogens of both medical and veterinary importance.

These recent advances in our understanding of the pathogenic importance of bartonellae to man and domesticated animals have not been matched by our understanding of the ecology of most *Bartonella* species. The only known vertebrate host of *B. bacilliformis* and *B. quintana* is human beings, whilst cats appear to be the reservoir host for both *B. henselae* and *B. clarridgeiae* [2]. Small mammals in Europe and North America are hosts to a large number of *Bartonella* species, although little information on pathogenicity in humans and host specificity is available [5, 6, 11]. Transmission between vertebrate hosts involves arthropod vectors for some species of *Bartonella*. For example, the nocturnal sandfly *Lutzomyia verrucarum* appears to be the vector for *B. bacilliformis*, and the body louse (*Pediculus humanus corporis*) is known to transmit *B. quintana* between humans [12, 13]. There is strong evidence for the role of the cat flea (*Ctenocephalides felis*) in the transmission of *B. henselae* between cats [14, 15], and fleas also appear to be important in the transmission of various rodent *Bartonella* species [16, 17].

The fastidious nature of bartonellae has led to a reliance on molecular methods for their identification and differentiation, and PCR-based approaches are widely used. Sequence data derived from analyses of DNA fragments have been used for assessment of the phylogenetic relationships of *Bartonella* species (e.g. 16S rRNA, citrate synthase, *groEL*). Although 16S rRNA gene sequence comparisons were initially used for these analyses, the DNA fragment for which data from the highest number of strains are now available is a 300–400 bp region at the 3' end of the citrate synthase gene (*gltA*). Phylogenetic reconstructions based on comparison of these sequence data have demonstrated well-supported topologies in which bartonellae isolated from rodents indigenous to the Old World (e.g. *Rattus*, *Clethrionomys*, *Mus*, *Sorex*, *Apodemus* sp.) cluster together. Furthermore, these Old World clades are divergent from clusters containing isolates from animals indigenous to the New World, including *Microtus pennsylvanicus*, as well as many sigmodontine rodents (e.g. *Peromyscus* and *Oryzomys* sp.) [18].

The observation that bartonellae isolated from *Rattus norvegicus* captured in North and South America possess genotypes very similar to those associated with *R. norvegicus* bartonellae in Europe has led to the hypothesis that rodents carried infection from

the Old World when introduced into the Americas [18]. This hypothesis was further supported by the isolation of a bartonella from a house mouse (*Mus musculus*) captured in the United States that clustered with bartonellae of other Old World rodents (*M. musculus* was also introduced into North America from Europe). To further evaluate the hypothesis that rodents can carry bartonellae between different geographic regions, we assessed the phylogenetic position of bartonellae isolated from UK and US populations of Eastern grey squirrels (*Sciurus carolinensis*). *Sciurus carolinensis* (referred to as 'grey squirrels' throughout the remainder of this manuscript) were first introduced into Britain in 1876 with 31 separate introductions noted by 1929; the majority of the introductions were between 1900 and 1920 [19]. We also characterized bartonellae isolated from native red squirrels (*Sciurus vulgaris*) to determine their evolutionary position and to investigate if transfer of bartonellae had occurred between the two sympatric species.

## MATERIALS AND METHODS

### Sampling of squirrels

In October 1998, red squirrels were live trapped at Formby Sands in northwestern England (53° 34·0' N, 3° 05·6' W) as part of a study on the health status of the population. Blood samples were collected from the femoral vein, and the squirrels were released at the site of capture. During the same period, blood samples were taken by cardiac puncture from grey squirrels captured from various sites on the Wirral peninsular in northwestern England and at Mere Sands (53° 38·1' N, 2° 50·2' W) where red squirrels were present until recently.

Grey squirrels trapped in Bulloch County, GA and New York, NY, USA, were collected originally for ectoparasite or zoonotic agent studies (hantavirus). In Bulloch County, GA, grey squirrels were live-trapped at three sites, including a private residence and the campus of Georgia Southern University in Statesboro, GA (32° 25·5' N, 81° 47·0' W) and Clito, GA (32° 30·8' N, 81° 45·2' W) from February through April 1997. Captures were anaesthetized with methoxyflurane, bled from the femoral vein, tagged for future studies and released at the site of capture. Grey squirrels from New York City, New York (40° 42·8' N, 74° 0·4' W) were collected in October 1996 for hantavirus studies. Blood samples were obtained from all

animals by cardiac puncture and animals were euthanised. Samples were frozen at  $-70^{\circ}\text{C}$  until used for this study.

### Isolation of *Bartonella* species

Isolation of *Bartonella* species followed methods described previously [11]. Briefly, isolates were obtained by streaking red blood cells onto Columbia blood agar plates supplemented with 5% horse blood (European isolates) or heart-infusion agar supplemented with 5% rabbit blood (US isolates). Plates were incubated at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for up to 14 days. Plates were checked daily for bacterial growth, and colonies tentatively identified as bartonella (based on colony morphology) were streaked onto clean plates. After 3–5 days' growth, colonies were harvested for PCR.

### DNA amplification and identification of *Bartonella* species

For samples from England, DNA was extracted from isolates by placing colonies into a microcentrifuge tube containing 500  $\mu\text{l}$  of sterile double distilled water. The mixture was homogenized thoroughly, boiled for 10 min, and then 5  $\mu\text{l}$  was used as DNA template for PCR. For DNA samples collected in North America, DNA was extracted using the QIAamp Blood Kit according to manufacturer's instructions (Qiagen, Inc., Chatsworth, CA, USA).

Two regions were targeted for PCR amplification, the citrate synthase (*gltA*) and the *groEL* heat-shock genes. An approximately 330 bp region of the *gltA* gene was amplified using the primers BhCS781.p and BhCS1137.n [20]. Amplification of an approximately 1400 bp of the *groEL* gene was achieved using the primer pair HS223 and HS1630 [21].

Products from the *gltA* and *groEL* PCR were purified using the Wizard PCR Preps system (Promega, Madison, WI, USA) according to the manufacturers instructions, then sequenced using an ABI 377 automated sequencer (Applied Biosystems Inc., Foster City, CA). Novel sequences were compared with other bartonellae in GenBank using the BLAST programme of the GCG computer software package (Wisconsin Sequence Analysis Package, Genetics Computer Group, version 8.1). Sequences were aligned using PILEUP (GCG). Using the phylogenetic package PHYLIP [22], SEQBOOT was used to create 100 multiple datasets for bootstrap analysis. Distance matrices were computed using the Kimura two-parameter

model (DNADIST). Phylogenetic trees were constructed using the neighbour-joining analysis programme NEIGHBOR and a consensus tree using *B. bacilliformis* as the outgroup was made using the CONSENSE programme. Trees were drawn using Treeview [23]. Additionally, maximum parsimony trees were produced using the GCG program DNAPARS, and again CONSENSE was used to produce a consensus tree.

### Sequence accession numbers

The *gltA* sequences of previously characterized *Bartonella* species used in the phylogenetic analyses have the following GenBank accession numbers: *B. bacilliformis* U28076, *B. clarridgeiae* U84386, *B. doshiae* AF207827, *B. tribocorum* AJ005494, *B. elizabethae* Z70009, *B. grahamii* Z70016, *B. taylorii* Z70013, *B. alsatica* AF204273, *B. birtlesii* Z70012, *B. quintana* Z70014, *B. koehlerae* AF176091, *B. henselae* L38987, *B. vinsonii arupensis* AF214557, *B. vinsonii* Z70015, *B. vinsonii berkhoffii* U28075, *B. sp.* MM5136ca AF086637, *B. sp.* sh6537ga U84377, *B. sp.* sh6397ga U84372, *B. sp.* pl7238nc U84379, *B. sp.* sh6396ga U84375. Novel isolates from this study have the following accession numbers: *B. sp.* 'Red' AF449760, *B. sp.* 'Grey' AF449761.

The *groEL* sequences used in the analyses included: *B. bacilliformis* Z15160, *B. clarridgeiae* AF014831, *B. doshiae* AF014832, *B. tribocorum* AF304018, *B. elizabethae* AF014834, *B. grahamii* AF014833, *B. taylorii* AF304017, *B. alsatica* AF299357, *B. birtlesii* AF355773, *B. quintana* AF014380, *B. henselae* AF014829, *B. vinsonii arupensis* AF304016, *B. vinsonii* AF014835, *B. vinsonii berkhoffii* AF014836. Squirrel isolates have the following accession numbers: *B. sp.* 'Red' AF449762, *B. sp.* 'Grey' AF449763.

## RESULTS

### Prevalence of bartonella in squirrels

In total, specimens from 20 grey squirrels and 20 red squirrels were analysed from the United Kingdom, while 15 and 3 grey squirrels from Georgia and New York, respectively, were available for isolation attempts.

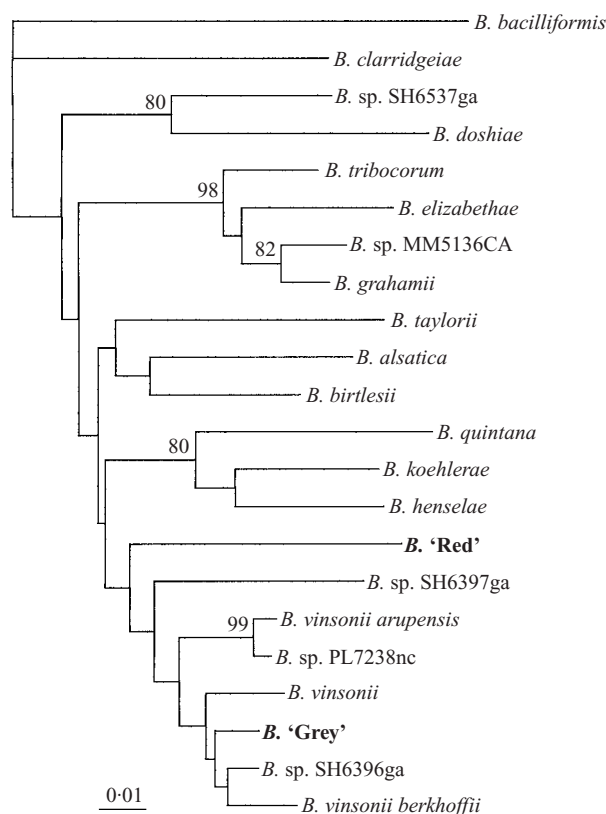
*Bartonella* was isolated from 12 of the 20 samples from red squirrels (60%) and 4 of 20 (20%) grey squirrels from the United Kingdom. Overall prevalence of infection in grey squirrels tested from the

United States was 28% (4 of 15 from Georgia and 1 of 3 from New York). Sequence data using the *gltA* primers indicated that there were two novel genotypes of bartonella, one each infecting red ('Red') and grey ('Grey') squirrels. All the red squirrel isolates were identical. The 5 grey squirrel isolates from the United States were identical to the 4 isolates from the United Kingdom. Novel sequences from the bartonellae obtained from red and grey squirrels were unlike any other bartonella sequences in GenBank.

Phylogenetic analysis of *gltA* sequences indicated that the novel genotype from grey squirrels ('Grey') grouped with or were most closely related to bartonella from other New World hosts, including *B. vinsonii* (from *Microtus* species; 97.5% similar), *B. vinsonii berkhoffii* (from a dog, 97% similar), and one *Sigmodon hispidus* (SH6396GA, U84375); 98.5% similar). The bartonella from the red squirrel ('Red') formed no significant grouping with any of the published sequences (Fig. 1), and was 91.5% similar to the bartonella from the grey squirrels. Although bootstrap values were generally low, the analysis produced two clades consisting exclusively of bartonellae from Old World hosts (Fig. 1), including one comprised of *B. tribocorum* (from a *Rattus norvegicus* [24]), *B. grahamii* (from *Clethrionomys glareolus*, *Microtus agrestis* and *Apodemus* species [11]), and *B. elizabethae* (from *Rattus norvegicus* [18]). Another clade consisting of bartonellae isolated exclusively from Old World rodents included *B. taylorii* (from *Clethrionomys glareolus* and *Microtus agrestis* [11]), *B. alsatica* (from the European rabbit, *Oryctolagus cuniculus* [25]), and *B. birtlesii* (from *Apodemus sylvaticus* [26]). Three of the six recognized pathogenic bartonellae (*B. quintana*, *B. koehlerae* and *B. henselae*) grouped together in one clade (Fig. 1).

Interestingly, the analysis indicated that *B. doshiae* (from a *M. agrestis* captured in the United Kingdom [11]) grouped with a bartonella isolated from a *Sigmodon hispidus* captured in Georgia, USA (SH6537GA).

Sequence analysis of the *groEL* PCR products showed a similar degree of divergence between the red and grey squirrel isolates, and again indicated 100% identity between the UK and US grey squirrel isolates. The phylogenetic analysis produced a tree with a similar topology to that of the *gltA* analysis (Fig. 2). The support for the cluster of 'Grey' genotype with *B. vinsonii* (95.9% similar), *B. v. arupensis* (96.8% similar) and *B. v. berkhoffii* (95.5% similar) was stronger than that shown by the *gltA* analysis. The 'Red' genotype was 91.3% similar to 'Grey' and was again

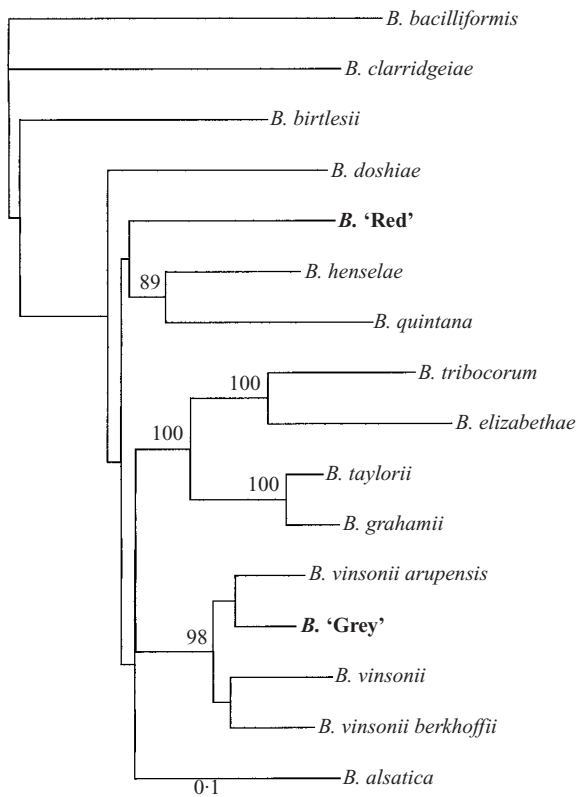


**Fig. 1.** Rooted neighbour-joining tree showing relationship of new bartonella isolates from red ('Red') and grey ('Grey') squirrels to previously described bartonella genotypes. Tree based on distances (Kimura) derived from 331 bp fragment of citrate synthase (*gltA*) gene. Bootstrap values > 75 (out of 100) are shown. *Bartonella bacilliformis* is the outgroup. All distances are shown to scale.

distinct from the other groups. In addition, the *groEL* analysis provided much better support for the clustering of New World isolates and one of the Old World clades suggested by the *gltA* analysis. However, the clade produced by the *gltA* analysis that included *B. birtlesii*, *B. taylorii* and *B. alsatica* was not reproduced in the *groEL* analysis, *B. taylorii* instead formed part of the clade containing *B. grahamii*, *B. elizabethae* and *B. tribocorum*. For both the *gltA* and the *groEL* sequences, analysis using the maximum parsimony method produced near-identical results to the distance analysis, with no difference in the clades shown in Figures 1 and 2 (trees not shown).

## DISCUSSION

This is the first report to describe the isolation and characterization of two novel bartonella from sciurid rodents: one from grey squirrels (*S. carolinensis*)



**Fig. 2.** Rooted neighbour-joining tree showing relationship of new bartonella isolates from red ('Red') and grey ('Grey') squirrels to previously described bartonella genotypes. Tree based on distances (Kimura) derived from 1206 bp fragment of *groEL* heat shock protein. Bootstrap values >75 (out of 100) are shown. *Bartonella bacilliformis* is the outgroup. All distances are shown to scale.

sampled in both the United States and the United Kingdom and one from red squirrels (*Sciurus vulgaris*) sampled in the United Kingdom. All isolates obtained from grey squirrels appeared identical, regardless of the capture location and only a single genotype was obtained from the red squirrels from the United Kingdom. The bartonella obtained from the Eastern grey squirrels were phylogenetically closest to *B. vinsonii*. While this species of *Bartonella* is not known to be a human pathogen, related subspecies have been implicated in causing disease in both domestic dogs and humans [27, 28]. Given the widespread distribution, common occurrence in urban areas, and peridomestic affinities of these small mammals throughout North America and Europe, additional investigations are warranted to examine the public health implications of these findings.

We found evidence that Old World and New World bartonellae are phylogenetically distinguishable. Analyses using the *gltA* gene showed that two

clades of bartonellae from Old World hosts grouped together (*B. taylorii*, *B. alsatica*, and *B. birtlesii* in one clade; *B. tribocorum*, *B. grahamii*, and *B. elizabethae* in another clade), while a large New World clade consisting of bartonella isolated from New World microtine (e.g. *B. vinsonii* from *Microtus* spp.) or sigmodontine rodents (e.g. PL7238NC from *Peromyscus leucopus*) was evident (Fig. 1). This was partly supported by the *groEL* analysis, where the New World clade was repeated but only one of the Old World clades was reproduced, and both *B. birtlesii* and *B. alsatica* appeared phylogenetically distinct from other bartonellae. Only with additional collection of isolates from New and Old World hosts and further molecular characterization of existing isolates will we be able to ascertain if these patterns are corroborated or simply the result of a small sample size. A larger sample of bartonellae from Old World hosts is particularly needed to address these questions.

Although these results are based on relatively small sample sizes from rodents collected in only a few places, the lack of diversity of *Bartonella* species in either host was nonetheless a surprising finding. Previous studies of rodent bartonellae in both the United States and the United Kingdom have shown a diverse community of *Bartonella* species present [5, 11, 29]. Studies suggest that fleas are important vectors in the transmission of at least some of these species [16, 17], and a number of flea species are associated with woodland rodents in the United Kingdom [30, 31]. However, squirrels have a comparatively restricted flea fauna – outside of Scotland the red squirrel is infested only by *Monopsyllus sciurorum* and the grey squirrel only by *Orchopeas howardi* [30, 31]. If fleas are the vectors of these bartonellae, the reason for the reduced diversity in bartonellae may be simply a result of lower vector diversity. Additional sampling from wild-captured squirrels over several seasons and controlled laboratory experiments are needed to address these hypotheses.

The discovery of apparently different bartonellae in the squirrel species present in the United Kingdom is potentially of further interest, as the red squirrel is the subject of intense conservation effort. It has previously been suggested that the expanding population of grey squirrels is one potential reason for the decline in red squirrels, either through direct interaction or as the result of some infectious agent. A parapoxvirus that appears highly pathogenic to reds but largely innocuous to greys has been suggested as one possible reason for the decline in red squirrel populations [32].

Similar to that seen in woodland rodents naturally infected with bartonella, no obvious clinical signs were seen in bartonella-infected squirrels. However, the potential of the 'Grey' genotype to be pathogenic to red squirrels is not known. Bartonellae have previously been shown to have pathogenic effects in atypical hosts, for example human infection with *B. henselae* and *B. clarridgeiae*, and a negative effect on host reproduction has been reported for house mice infected with *B. birtlesii* [33], a species naturally found in British woodland rodents [26]. Controlled laboratory studies should be conducted to show if these species of *Bartonella* are host specific and if infection with these bartonellae causes reduction in fitness of the host.

It would be of particular interest to study sympatric populations of squirrels to determine whether there is any transfer of bartonellae in nature, and to also obtain samples from a wider geographic range to establish whether there is any diversity among the bartonellae infecting red and grey squirrels in both the United States and the United Kingdom. The study of other mammalian species that have been introduced into the United Kingdom from the United States, such as mink (*Mustela vison*) would also provide further information on the ability for hosts to transfer bartonellae from one geographic region to another.

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