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Description of *Bartonella bennetti* sp. nov., a novel rodent-associated species, with comparative genomics of the *Bartonella* genus

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Abstract

The genus *Bartonella* comprises over 40 species, most of which are haemoparasites of mammals. Herein, we describe *Bartonella bennetti* sp. nov., a novel member of the genus, isolated from field voles (*Microtus agrestis*) in Kielder Forest, UK. Polyphasic characterization of three strains (C271^T, D105 and J117) of the proposed species indicated that they were closely related to members of phylogenetic lineage 3 (L3) of the genus. The average nucleotide identity (ANI) between C271^T and other L3 species ranged between 88.8 and 90.6%, supporting the proposal of a new species. C271^T shared ANIs approaching 96% with other members of L3 that are yet to be validly published but exhibited marked genomic, ecological and biogeographical differences from them, further justifying the creation of a new taxon. All three *B. bennetti* sp. nov. strains were found to possess genes encoding three VirB/D4 type IV secretion systems and associated effector proteins and to harbour a chromosomally integrated F-type conjugative plasmid, which forms an Hfr-like configuration not previously observed in the *Bartonella* genus. This integration could facilitate large-scale chromosomal gene transfer during conjugation, with potential consequences for adaptation, recombination and niche differentiation. The phylogenetic structure of L3, coupled with the ecological partitioning of its members, suggests that covert host specificity, not generalism, is the dominant mode of diversification. The absence of the Trw system, which facilitates host switching in lineage 4 (L4), may constrain ecological breadth in L3, thought to be undergoing a parallel adaptive radiation with L4. The discovery of *B. bennetti* sp. nov. underscores the importance of combining genomic, ecological and evolutionary evidence when delimiting species boundaries in *Bartonella* and raises new hypotheses about the role of Hfr-mediated recombination in the evolutionary dynamics of host-adapted pathogens. The type strain of *B. bennetti* sp. nov. is C271^T (CSUR B1113^T, NCTC 15117^T).

INTRODUCTION

The genus *Bartonella* currently comprises over 40 validly published species together with many *Candidatus* taxa and partially characterized strains [1]. The evolutionary history of the genus is marked by a remarkable transition from insect symbiosis to mammalian parasitism. The species associated with mammalian parasitism, termed eubartonella, are haemotrophic and arthropod transmitted and form a large monophyletic group that has diverged from taxa considered gut-associated symbionts of social insects [2, 3]. *Bartonella* species parasitize a wide range of mammals, exploiting them as reservoir hosts through chronic infection of the vascular endothelium and erythrocytes, thereby facilitating their transmission to blood-feeding arthropod vectors [4]. Many bartonellae appear to be adapted to a single mammal species, but some are more generalist, exploiting several, often closely related, mammalian species [5]. Adaptation to reservoir host by bartonellae provides a clear indication of ecological distinction between taxa that is rare amongst bacteria; however, accommodating both ecology and 'classical' polyphasic characterization when defining *Bartonella* species is challenging and has resulted in inconsistency across the genus. The ecological integrity of

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Abbreviations: ANI, average nucleotide identity; CDS, protein coding gene; L3, lineage 3; L4, lineage 4; ONT, Oxford Nanopore Technologies; SEM, scanning electron microscopy; T4SS, type IV secretion system.
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Bartonella bennetti sp. nov. whole-genome accessions: C271^T, GCA_045945445.1; D105, GCA_050931875.1; and J117, GCA_050932065.1. *Bartonella bennetti* sp. nov. 16S rRNA accession: C271^T, OR452865.1. *Bartonella heixiaziensis* whole-genome accession: RE21, GCA_046245125.1.
Two supplementary tables and four supplementary figures are available with the online version of this article.

some species has been challenged [6–8], and thus, they may well represent a consortium of ecologically distinct organisms that are similar enough in genetic or genomic terms to be considered the same taxon using current thresholds widely applied for bacterial species delineation. Conversely, elsewhere in the genus, ecologically distinct organisms that possess genetic and genomic similarities akin to strains of the same species lie in different taxa [9, 10].

Phylogenetically, the eubartonellae form four deep-branching lineages (L1–L4) [11, 12], and this radiation is reflected in the evolution of diverse macromolecular systems that mediate host cell interaction and immune evasion. Chief amongst these are type IV secretion systems (T4SSs), which play a central role in host interaction. Three major T4SSs, VirB/D4, Vbh and Trw, have been identified in the genus, each exhibiting distinct structural and functional properties [11]. The VirB/D4 system, present in L3 and L4, secretes bartonella effector proteins (Beps) that modulate host cell physiology and facilitate persistent infection [12, 13]. The Vbh system, a homologous variant, is variably encoded on plasmids or the chromosome and may function either in interbacterial interactions or as a conjugation system, although many chromosomal versions appear to be degenerate [14]. In contrast, the Trw system is restricted to L4 species and mediates erythrocyte adhesion, replacing ancestral flagella and enabling species-specific host cell binding [11, 15].

Eubartonella L3 currently contains two validly published species, *Bartonella clarridgeiae*, which is adapted to cats [16], and *Bartonella rochalimae*, the ecology of which is currently uncertain [17–19]. The lineage also contains a newly described but not yet validly published species '*Bartonella bilalgolemii*' [20] as well as a number of other partially characterized bartonellae. Amongst these are strains referred to as 'BGA' or '*B. rochalimae*-like', primarily defined on the basis of partial *gltA* nucleotide sequence similarity, parasitizing various rodent species across Europe [21–23]. Given their wide distribution and apparent adaptation to various rodent species, these '*B. rochalimae*-like' strains may well possess different ecologies and thus their taxonomic integrity is unclear. Here, we report the polyphasic characterization of three *Bartonella* strains previously identified as 'BGA'/'*B. rochalimae*-like': C271^T, D105 and J117 [21], all isolated from field voles (*Microtus agrestis*) in Kielder Forest, Northumberland, between 2001 and 2004 [21]. Characterization of the isolates supports their accommodation in a novel L3 species, for which we propose the name *Bartonella bennetti* sp. nov.

METHODS

Bacterial isolation and culture

Strains C271^T, D105 and J117 were isolated from the blood of field voles (*M. agrestis*) trapped in Kielder Forest (55.2344° N 2.5791° W), which lies on the eastern edge of the border between England and Scotland between 2001 and 2004, as previously described [21]. In addition, we characterized the RE21 strain of *Bartonella heixiaziensis*, isolated from a field vole in Assynt Forest, North-West Scotland (58.215° N 5.0505° W) in July 2021. We included this isolate as no representative whole-genome sequence for the species was available. All strains were preserved in brain heart infusion broth containing 10% (v/v) glycerol at −80 °C.

Isolates were revived on Columbia agar supplemented with 10% (v/v) whole horse blood and incubated at 35 °C in a 5% CO_2 atmosphere. We assessed the ability of *B. bennetti* sp. nov. strain C271^T to grow in microaerophilic (8–9% O_2; 7–8% CO_2) and anaerobic (<1% O_2; 9–11% CO_2) conditions at 35 °C on 10% horse blood-enriched Columbia agar. Atmospheric concentrations were achieved in 2.5 l containers with the ThermoFisher Scientific Oxoid™ CampyGen™ 2.5 l sachet (CN0025A) and the Oxoid™ AnaeroGen™ 2.5 l sachet (AN0025A), respectively. The Oxoid™ Resazurin Anaerobic Indicator strips (BR0055B) were used to verify the presence or absence of oxygen in the sealed containers.

For scanning electron microscopy (SEM), colonies of strains C271^T, D105 and J117 were sampled by placing sterile glass coverslips directly onto colonies growing the agar surface, followed by gentle removal and rinsing in PBS to eliminate residual media. Subsequent fixation, post-fixation, dehydration, drying and sputter coating were carried out following the protocol described by Fischer (Basic Protocol 1) [24]. Prepared samples were imaged at the Scanning Electron Microscopy Shared Research Facility (SEM SRF), University of Liverpool, UK.

Biochemical characterization

For biochemical and metabolic characterization, strains C271^T, D105 and J117 were tested with the 20NE (BioMérieux) identification system, following the manufacturers' protocol. Additionally, all strains were tested for catalase and oxidase activity and subjected to Gram staining.

Genome sequencing and assembly

Genomic DNA extractions were performed on strains C271^T, D105 and J117 using the Wizard® HMW DNA extraction kit (Promega: A2920) following the manufacturers' protocol. The genomes were sequenced using both Illumina NovaSeq (2×250 bp paired-end) and Oxford Nanopore Technologies (ONT) MinION (LSK109/R9.4.1) platforms. For Illumina sequencing, 100 ng of DNA in 100 µl was dispatched to MicrobesNG. Genomic libraries were then prepared by MicrobesNG (Birmingham, UK) using the Nextera XT Library Prep Kit following the manufacturers' protocol with the following modifications: input DNA increased

twofold, and PCR elongation time increased to 45 s. ONT libraries were prepared in-house with SQK-LSK109 and barcoded with the native barcoding expansion 1–12 (EXP-NBD104) according to the manufacturers' protocol. A MinION R9.4.1 flow cell was loaded on the MK1C system running minKNOW 22.08.9 with basecalling set to fast.

Whole-genome sequencing of *B. heixiaziensis* RE21 was outsourced to MicrobesNG. Short-read sequencing matched that described for the *B. bennetti* sp. nov. strains. ONT long-read sequencing was performed using 400–500 ng of HMW DNA and the SQK-LSK109 kit with Native Barcoding EXP-NBD104/114 on an R9.4.1 flow cell in a GridION.

All raw short-read and long-read datasets were processed as follows: raw short reads (2×250 bp paired-end) were trimmed with FastP [25] with default parameters and Q-score set for 20. Raw long-read data was concatenated, and adapters were removed with Porechop [26] and filtered to exclude all reads less than 1,000 bp with Filtlong (GitHub rrwick/Filtlong). Assembly of all four strains was executed by the Unicycler (0.5.0) hybrid assembly pipeline [27]. The genomes were ultimately annotated using the RASTtk pipeline [28].

Phylogenetic analysis

A whole-genome phylogenetic tree was reconstructed using RAxML [29] implemented into the BV-BRC [30]. The analysis included all species of the genus *Bartonella* with validly published names, together with partially characterized strains and all *Candidatus* bartonellae with complete genomes (Table S1, available in the online Supplementary Material). A total of 500 single-copy genes were identified for the analysis totalling 178,160 aligned amino acids from 53 genomes. Protein sequences were aligned with MAFFT [31], and the DUMMY2 protein substitution model was used to define amino acid frequencies. Branch support was calculated with the RAxML fast bootstrapping methodology, and *Brucella abortus* strain NCTC 624 (GCA_900446015.1) was used as the outgroup.

A second whole-genome tree was reconstructed using RAxML implemented into the BV-BRC focusing on L3 *Bartonella* species and strains. A complete list of strains and species used for the analysis is available (Table S2).

Average nucleotide identity analysis

Whole-genome average nucleotide identity (ANI) was calculated between the closest known relatives of each strain based on whole-genome phylogenetic analyses: for the *B. bennetti* sp. nov. strains C271^T, D105 and J117, *B. rochalimae*, *B. clarridgeiae*, '*B. bilalgolemii*', AR15-3, JB15, CDCSkunk and Coyote22sub were chosen. For RE21, *Bartonella jaculi*, '*Bartonella gliris*' and *Ca*. Bartonella washoensis were identified as close relatives. Finally, representatives for each remaining bartonellae lineage, *Bartonella bacilliformis* (L1) and *Bartonella capreoli* (L2), were selected. ANI was calculated with OrthoANIu [32] available online (last accessed on 27 March 2025: https://www.ezbiocloud.net/tools/ani).

Plasmid analysis

A complete list of plasmids available on NCBI GenBank was compiled and subjected to ANI analysis with OrthoANIu [32]. The analysis included reconstructed plasmids from chromosomally integrated elements.

RESULTS AND DISCUSSION

Phenotypic and genotypic characterization

Colonies of all *B. bennetti* sp. nov. strains (C271^T, D105 and J117) were indistinguishable from one another and were small, smooth and white after 5 days of growth at 35 °C in a 5% CO_2 atmosphere, with early growth detectable at 3 days. Strain C271^T also grew well at 35 °C under micro-aerophilic (8–9% O_2; 7–8% CO_2), but not anaerobic (<1% O_2; 9–11% CO_2), conditions. Gram staining of colonies revealed all strains to be indistinguishable Gram-negative rods. Under SEM, strains C271^T, D105 and J117 were indistinguishable, appearing as small rods 0.8–1.2 µm in length and 0.3–0.4 µm in width with up to four polar lophotrichous flagella measuring up to 2 µm in length visible on a single bacterium (Fig. 1). All three *B. bennetti* sp. nov. strains were biochemically inert in the tests we performed. They were unable to reduce nitrates, produce indole, ferment glucose or utilize arginine or citrate. They did not have any detectable beta-galactosidase, catalase, gelatinase, oxidase or urease activity. They were unable to assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate or phenylacetic acid. All strains were found to be catalase and oxidase negative. Thus, on the basis of all phenotypical characterizations performed, *B. bennetti* sp. nov. could not be distinguished from the other L3 species with validly published names, *B. clarridgeiae* or *B. rochalimae*. The inability of classical phenotypic testing to distinguish between *Bartonella* species is widely recognized [33].

Although the *B. bennetti* sp. nov. strains could not be distinguished from another or from other L3 *Bartonella* species using phenotypic characterization techniques, there were some genomic variations between the strains (C271^T, D105 and J117) and between species. Most notably, variation in genome length and predicted number of protein-coding genes (CDS), with the

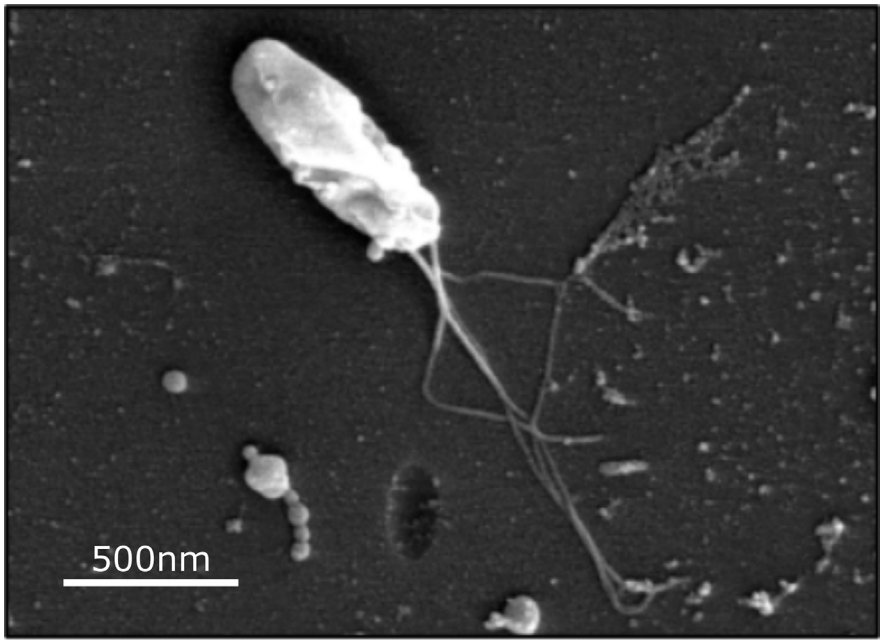

Fig. 1. SEM of *B. bennetti* sp. nov. strain C271^T. A 500-nm scale bar is shown within the image (80,000×).

genomes of *B. bennetti* sp. nov. strains being about 100 kB larger than those of *B. clarridgeiae* and *B. rochalimae* and possessing at least 100 extra CDS (Table 1).

Phylogenetic placement and genomic relationships

The phylogenetic tree indicated that *B. bennetti* sp. nov. was an L3 species closely related to *B. rochalimae*, *B. clarridgeiae* and '*B. bilalgolemii*' (Fig. 2). RE21 was determined to be closely related to *B. heixiaziensis* CR90HXZ^T through comparisons of the 16S rRNA gene (1267 bp) (KJ361623.1) and the *gltA* gene (326 bp) (KJ175047.1), utilizing the MUSCLE alignment tool [34] integrated into Geneious Prime (11.0.18). RE21 shared 100 and 98.92% similarity at these loci, respectively. Subsequent phylogenetic analysis confirmed the identification of RE21 and placed *B. heixiaziensis* RE21 into L4 clustering with *B. jaculi*, *Ca*. *B. washoensis* and '*B. gliris*' (Fig. 2).

The topology of the L3-focused tree corroborated the genus tree, identifying the partially characterized strain AR15-3 and '*B. bilalgolemii*' as the closest relatives of *B. bennetti* sp. nov. (Fig. 3).

The ANI between *B. bennetti* sp. nov. C271^T and the partially characterized strain *Bartonella* sp. AR15-3 was 96.04% across 61.23% of the genome, and the ANI between *B. bennetti* sp. nov., C271^T and '*B. bilalgolemii*' G70 (not validly published under the ICNP) was 95.98% across 67.52% of the genome (Table 2). The ANI values amongst *B. bennetti* sp. nov. strains were >99.6%.

Table 1. Genome statistics for *B. bennetti* sp. nov. (strains C271^T, D105 and J117) and lineage 3 *Bartonella* species: *B. rochalimae* BMGH^T and *B. clarridgeiae* 73

***Bartonella* strain**	**C271^T**	**D105**	**J117**	***B. rochalimae* BMGH^T**	***B. clarridgeiae* 73**
NCBI assembly/accession	CP174576.1	CP194514.1	CP194513.1	GCA_000706645.1	GCA_000253015.1
Estimated chromosome length (bp)	1,629,655	1,657,696	1,643,736	1,534,143	1,522,743
DNA G+C content (mol%)	35.9	36.0	35.9	35.7	35.7
Protein-coding genes (CDS)	1,555	1,609	1,506	1,397	1,334
tRNA	41	44	41	42	42
rRNA	6	9	6	6	6
Number of contigs (scaffolds)	1 (circular)	1 (circular)	1 (circular)	19 (3)	1 (circular)

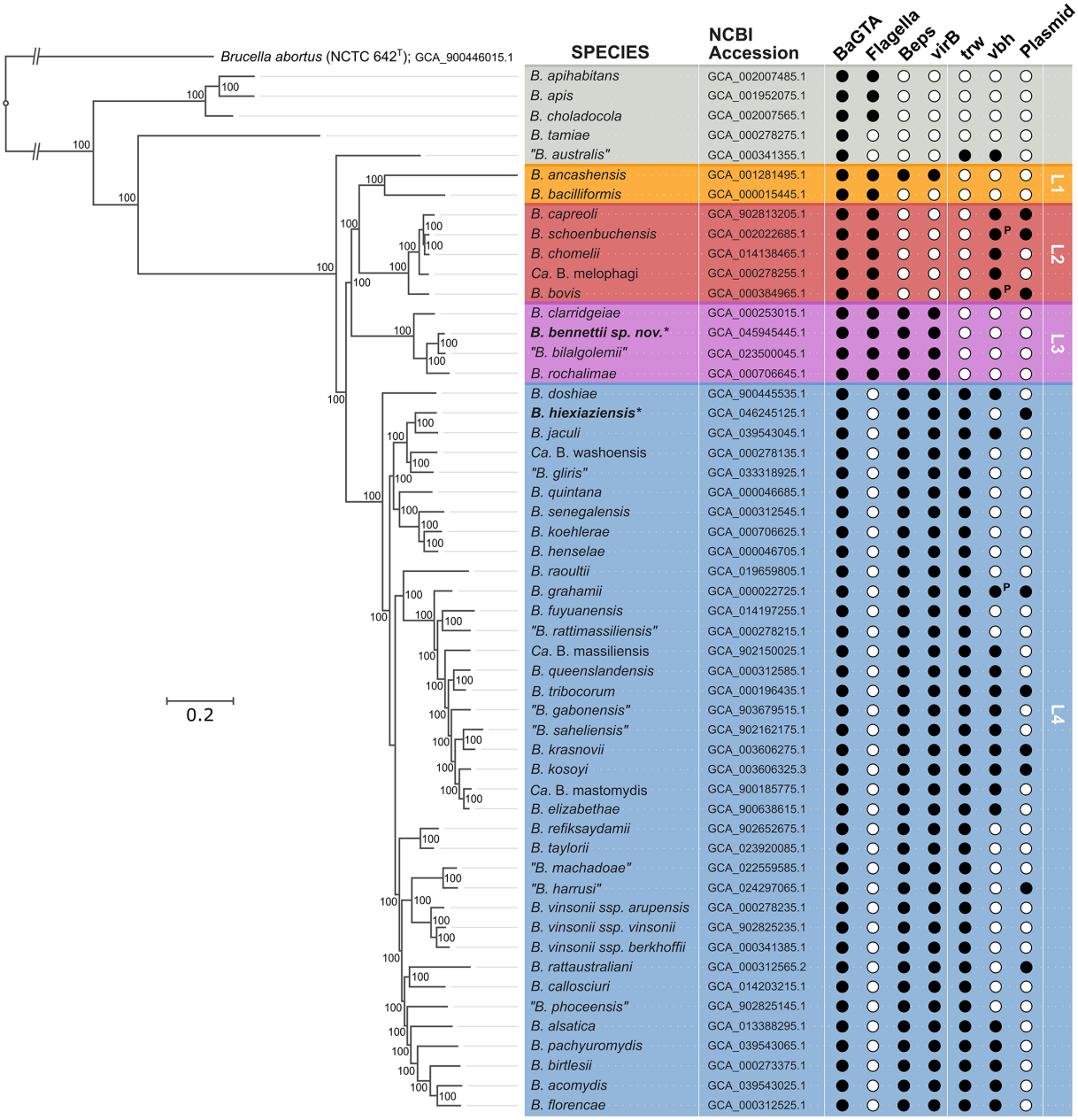

Fig. 2. Core genome tree of 53 *Bartonella* taxa and *B. abortus* (NCTC 642^T) as the outgroup. A 500-gene maximum-likelihood tree with 10 allowed duplication and deletion to increase resolution was generated with RAxML from an alignment of 625,854 amino acids and bootstrapped with 100 replicates. The presence and absence of key virulence factors are indicated in columns. P indicates that the Vbh T4SS is present on a plasmid. Species characterized in this study are marked with (*); *Ca.* denotes *Candidatus* status; taxa in quotation marks are not validly published. NCBI accessions (see Table S1 for more information).

B. heixiaziensis RE21 shared an ANI of 88.95% across 54.76% of the *B. jaculi* genome, identifying *B. jaculi* as the closest known relative of *B. heixiaziensis* RE21. These results corroborate the whole-genome phylogenetic analysis (Fig. 2).

Virulence factor distribution and analysis

In addition to phylogenetic placement, the presence and absence of genes encoding key bartonellae-associated virulence factors were recorded for all genomes (Fig. 2). These included genes encoding the bartonella gene transfer agent (BaGTA) [35], flagella and three distinct T4SSs: VirB/D4, Trw and Vbh. Genes encoding the Trw T4SS and flagella were mutually exclusive across the genus, with the Trw operon present only in (all) L4 taxa and the divergent species *Bartonella australis*. Genes encoding the VirB/

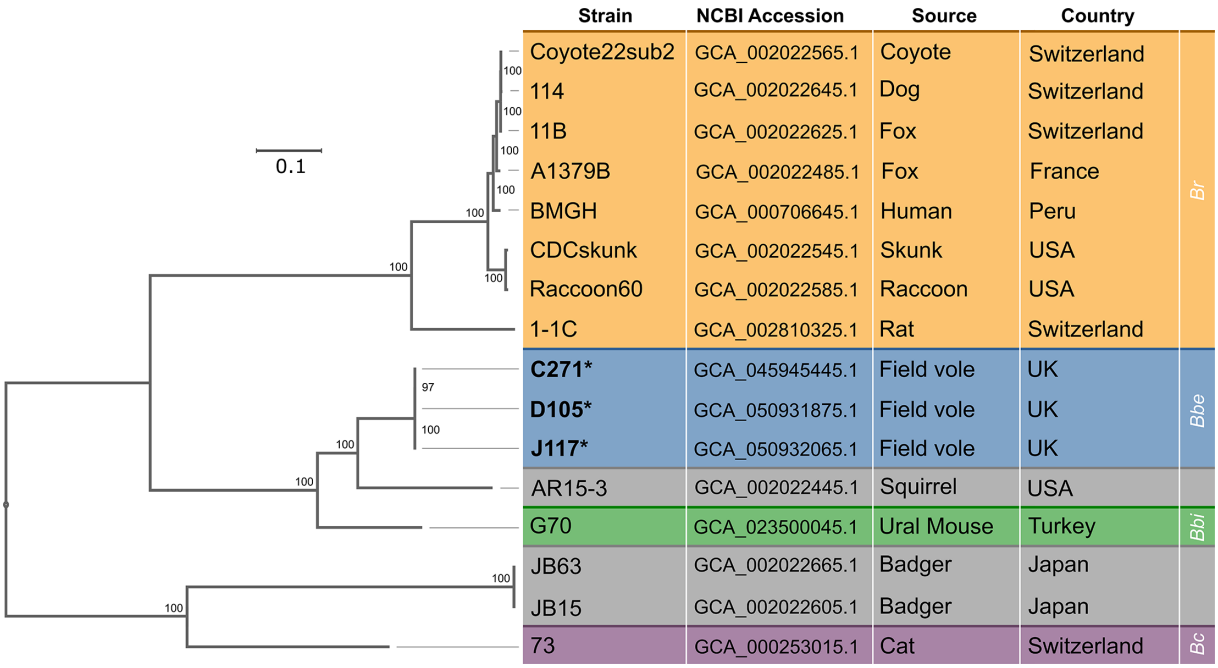

Fig. 3. Phylogenetic tree of lineage 3 *Bartonella* strains including the type strains of *B. rochalimae* (*Br*) and *B. clarridgeiae* (*Bc*) and '*B. bilalgolemii*' (*Bbi*). Strains C271^T, D105 and J117 are proposed as a novel lineage 3 species *B. bennetti* sp. nov. (*Bbe*). A tree was generated with RAxML using 619,662 amino acid alignments of 500 core single-copy genes for each species with an allowance for 10 duplications and deletions to enhance resolution, bootstrapped with 100 replicates. Strains from this study are marked with * (see Table S2 for more information).

D4 and Vbh secretion systems were more sporadically distributed across the genus; *virB/D4* operons are present in all L3 and L4 species and the L1 species *Bartonella ancashensis*, indicating at least three separate acquisition events. The *vbh* operon is present in 23 species either on a plasmid (3 instances) or within the bacterial chromosome (20 instances) (Fig. 2). The distribution of the *vbh* operon amongst *Bartonella* species suggests at least seven independent acquisition events; however, given its frequent association with plasmids, it is likely that additional, undetected acquisition events have occurred. Neither *B. bennetti* sp. nov. nor *B. heixiaziensis* possessed the *vbh* operon.

The *B. bennetti* sp. nov. genome contains three *virB/D4* operons (Fig. 4). The first copy of the operon is highly conserved across L3 species and strains (Fig. S1). *B. rochalimae* is the only species not to possess an associated *bep* and to have two small uncharacterized CDSs in its place. The second and third copies of the *virB/D4* operon are co-located, flanking a central *virD4* coupling protein gene and several *beps* (Fig. S2). The operons themselves can be found between *gltA* copies and show increased genetic reorganization compared to the first copy. The second and third copies of the *virB/D4* operon in C271^T, J117 and AR15-3 are identical in structure, but in D105, the operon is inverted between the flanking hypothetical CDS and *gltA*. *B. clarridgeiae* also contains a second and third *virB/D4* operon, but the central *bep* genes, *virD4* and flanking *gltA* genes are markedly rearranged, and it possesses an additional *bep* and a duplicate of the small hypothetical CDS present between *beps* in the *B. bennetti* sp. nov. strains. More substantial variation was observed in the architecture of the *virB/D4* operons in the *B. rochalimae* genome, with all but *virB2* and *virB3* of the third operon absent.

Chromosomally integrated F-plasmid and implications for horizontal gene transfer

A putative operon encoding a fourth secretion system, containing the *trb* and *tra* conjugative transfer gene clusters, was located in the *B. bennetti* sp. nov. genomes (Fig. 4). The genes present are indicative of an F-type conjugative plasmid that has been chromosomally integrated. The system was, however, absent from the genome of other L3 taxa or species elsewhere in the genus (Fig. S3), suggesting potentially recent acquisition and enabling the estimation of a plasmid size of 30,586 bp. We have named this chromosomally integrated plasmid pRecC271-1 to indicate it does not exist independently within the bacterium but rather is a reconstruction of a past plasmid that has undergone chromosomal integration.

B. heixiaziensis strain RE21 possesses a 29,248 bp plasmid (pRE21-1) containing 39 putative CDSs (Fig. S4). These include plasmid replication protein *repC*, plasmid partitioning protein *parA* and conjugal protein genes (*trb*, *tra*), characteristic of a fertility conjugative plasmid (F-plasmid) akin to pTLV-1, pOE11-1 and pBHa, present in *Bartonella kosoyi*, *Bartonella krasnovii* and '*Bartonella harrusi*', respectively [36].

Table 2. ANI and coverage of four strains of bartonellae: C271^T, D105, J117 (*B. bennetti* sp. nov.) and RE21 (*B. heixiaziensis*) against their closest relatives as well as representatives of all recognized bartonellae lineages (1–4) (quotation marks represent species currently without a valid publication according to the ICNP)

Reference genome (NCBI accession)	C271^T		D105		J117		RE21	
	OrthoANIu (%)	Coverage (%)	OrthoANIu (%)	Coverage (%)	OrthoANIu (%)	Coverage (%)	OrthoANIu (%)	Coverage (%)
B. rochalimae (GCA_000706645.1)	90.60	61.70	90.78	58.17	90.67	59.20	78.87	38.07
B. clarridgeiae (GCA_000253015.1)	88.82	58.30	88.72	58.80	88.84	59.00	79.20	37.82
'B. bilalgolemii' (GCA_023500045.1)	95.98	**67.52***	**96.04***	62.85	**96.09***	**67.05***	79.11	37.32
AR15-3 (GCA_002022445.1)	**96.04***	61.23	95.80	**70.93***	95.98	59.46	78.99	37.58
JB15 (GCA_002022605.1)	88.16	57.86	88.09	56.01	88.24	56.22	79.22	34.31
CDCSkunk (GCA_002022545.1)	90.71	58.91	90.72	62.48	90.68	61.45	79.23	36.37
Coyote22sub2 (GCA_002022565.1)	90.91	58.33	90.83	62.33	90.69	64.90	78.82	36.36
B. jaculi (GCA_039543045.1)	79.06	48.36	79.21	49.35	78.98	48.37	**88.95***	**54.76***
***Ca.* B. washoensis** (GCA_000278135.1)	79.29	47.83	79.24	46.46	79.27	47.66	87.50	50.47
'B. gliris' (GCA_045277155.1)	79.35	48.31	79.74	46.35	79.29	49.11	88.11	51.52
B. capreoli (GCA_902813205.1)	79.50	48.97	79.77	46.82	79.63	48.77	79.92	36.81
B. bacilliformis (GCA_009498695.1)	78.76	45.08	79.23	41.77	78.90	43.35	79.15	33.83

*Marks the highest score for each strain.

Comparative sequence analysis of pRecC271-1 and pRE21-1 with other (non-cryptic) plasmids associated with *Bartonella* species indicated between 0–50.98% and 5.28–63.32% sequence coverage, respectively (Table 3). The ANI scores on the covered sequence ranged from 76.49 to 86.40% for pRecC271-1 and from 76.62 to 85.40% for pRE21-1 (Table 3). Examination of the pattern of both the coverage and ANI values amongst bartonellae-associated plasmids revealed two distinct groups; the first of these comprised F-plasmids (pTLV-1, pOE11-1, pBHa, pRE21-1 and pRecC271-1), and the second comprised plasmids (pBT, pBGR3, pML and pNH4) carrying the *vbh* T4SS operon in *Bartonella tribocorum*, *Bartonella grahamii*, *Bartonella schoenbuchensis* and *Bartonella rattaustraliani* (Table 3).

Plasmid-mediated gene acquisition plays a pivotal role in the evolutionary divergence of *Bartonella* species, acting as a major driver of their adaptability and host specificity [35, 37]. Plasmids, such as pBGR3 in *B. grahamii* and PML in *B. schoenbuchensis*, carry *vbh* operons, illustrating the role plasmids have played in disseminating VirB/Vbh/Trw T4SS across the *Bartonella* genus [37, 38]. We encountered the relatively recent (i.e. species-specific) chromosomal integration of a classical F-plasmid into the *B. bennetti* sp. nov. genome. F-plasmids such as pTLV-1, pOE11-1, pBHa and pRE21-1 are commonly found in the *Bartonella* genus and represent a well-characterized class of conjugative plasmids, originally described in *Escherichia coli* [39, 40]. Their primary function is to facilitate horizontal gene transfer of the plasmid itself into recipient cells via a T4SS encoded by *tra* and *trb* genes [41]. Typically, this process involves transfer of the plasmid's own DNA, including the T4SS operon and any accessory genes it carries. However, when the F-plasmid integrates into the host chromosome, as has occurred in *B. bennetti* sp. nov., the strain becomes an Hfr (high-frequency recombination) donor [41–43]. In this state, gene transfer begins at the *oriT* site, now embedded in the chromosome and proceeding linearly into adjacent chromosomal DNA [41]. Although conjugation is frequently interrupted before full transfer, studies in *Pseudomonas putida* have shown that up to 23% of the chromosome could be transferred [44]. Given our discovery of further evidence of F-plasmids amongst *Bartonella* species and of their chromosomal integration and hence the potential induction of an Hfr state, it is not unreasonable to propose their importance in horizontal gene transfer within the genus. High-frequency recombination could facilitate large-scale exchange of genetic material amongst bartonellae co-infecting a host or vector, potentially spreading traits associated with virulence, antibiotic resistance or host switching. This mechanism could accelerate diversification and adaptation, promoting niche differentiation and host specificity within coexisting

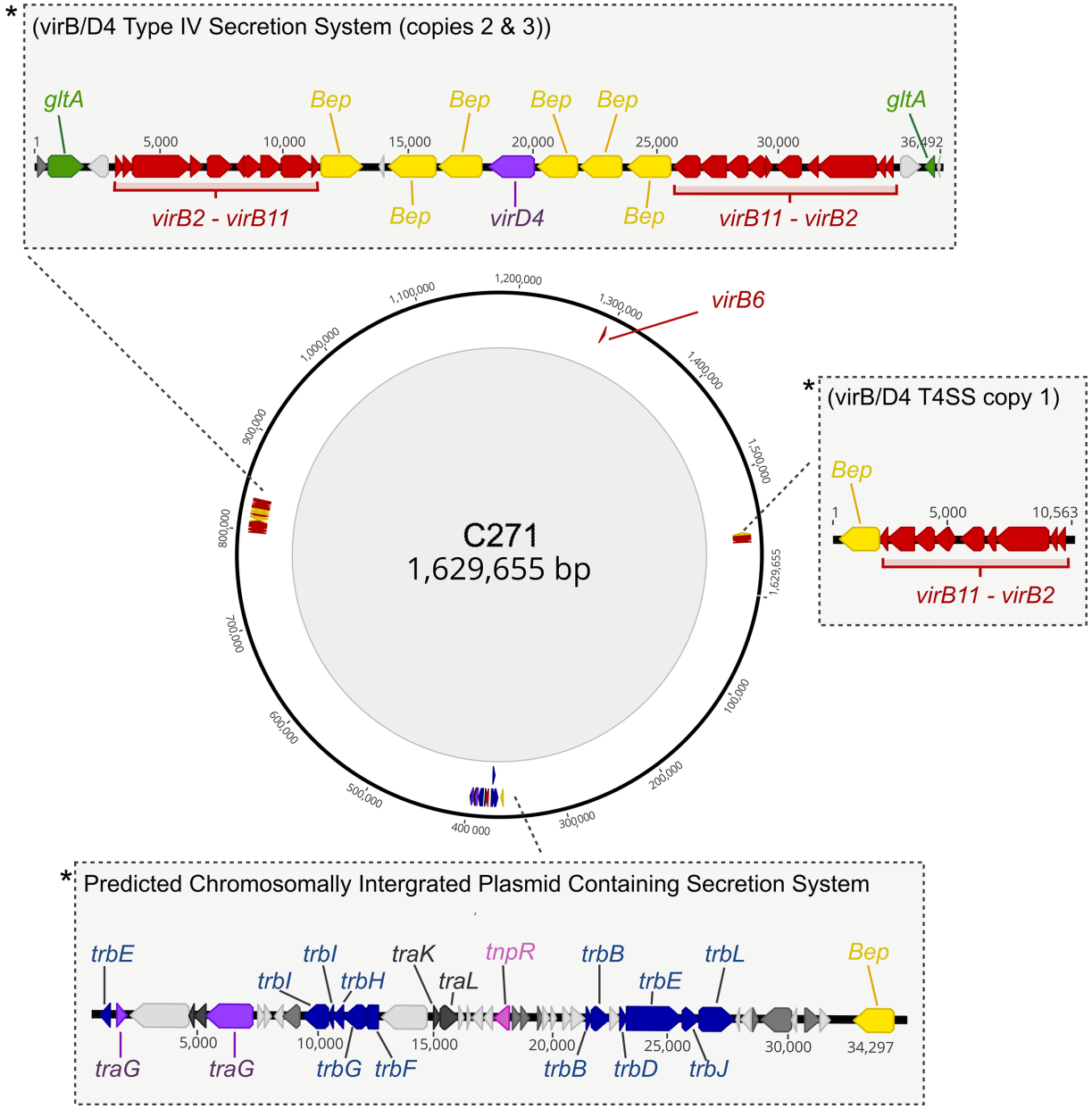

Fig. 4. Complete chromosome assembly of *B. bennetti* sp. nov. strain C271^{T} (NCBI accession: GCA_045945445.1). Sites of interest are highlighted, including (1) two copies of the *virB/D4* operon, (2) a third copy of the *virB/D4* operon and (3) a predicted chromosomally integrated plasmid. The image was generated using the Geneious software package.

populations. Furthermore, Hfr conjugation might help overcome genetic barriers by transferring chromosomal segments beyond the plasmid itself, thereby increasing genomic plasticity and fostering evolutionary innovation within a genus where low levels of genomic plasticity would be expected.

Species delimitation and ecological considerations

Species of bacteria are commonly defined through a combination of phenotypic, ecological and genomic criteria, with ANI widely used as a genomic proxy for species boundaries. A threshold of 95–96% ANI is often applied across bacterial genera [45, 46]. Although ANI has been proposed as a criterion for the taxonomic review of the *Bartonella* genus [47], ANI thresholds alone provide only a partial basis for species delineation. In the *Bartonella* genus, species boundaries are more accurately resolved by incorporating a polyphasic approach that incorporates ecological factors such as host specificity and geographic distribution. For example, *B. schoenbuchensis* and *Bartonella chomelii* exhibit borderline ANI values (96.81%) yet are considered distinct

Table 3. ANI of plasmid p-RE21-1 sequenced in *B. heixiaziensis* strain RE21 and a reconstructed plasmid pRecC271-1 from *B. bennetti* sp. nov. C271^T

Strains were compared to existing bartonellae plasmids and each other using OrthoANIu. *pRec*, plasmid reconstruction from the bacterial chromosome.

Reference plasmid (NCBI assembly)	Length (bp)	pRecC271-1		pRE21-1	
		OrthoANIu (%)	Coverage (%)	OrthoANIu (%)	Coverage (%)
***B. tribocorum* pBT** (AM260524.1)	23,343	0.00	0.00	80.25	5.28
***B. grahamii* pBGR3** (CP001563.1)	28,192	80.90	1.54	80.89	10.98
***B. schoenbuchensis* pML** (CM001845.1)	57,959	85.10	4.10	76.72	14.52
***B. rattaustraliani* pNH4** (FJ605483.1)	11,227	86.40	1.41	85.40	5.30
***B. kosoyi* pTLV-1** (CP042964.1)	29,057	77.76	50.08	83.11	51.40
***B. krasnovii* pOE11-1** (CP042965.1)	41,483	76.55	46.69	83.51	50.24
***'B. harrusi'* pBHa** (CP101113.1)	29,892	76.49	50.98	84.10	63.32
pRE21-1/pRecC271-1	29,248/30,586	78.58	48.26	78.58	49.98

species due to differences in host association (deer versus cattle). A similar pattern is observed between *B. bennetti* sp. nov., *'B. bilalgolemii'* G70 which is not validly published under the ICNP, and the unclassified strain *Bartonella* sp. AR15-3, which clusters near the 94–96% ANI threshold but likely exploits distinct host species across separate geographic regions: field voles in the UK [21], Ural field mice (*Apodemus uralensis*) in Turkey [20] and red squirrels (*Tamiasciurus hudsonicus*) in the USA, respectively. *B. bennetti* sp. nov. infections were detected in ~1.2% of nearly 4,000 field voles surveyed between September 2001 and September 2004 [21], but in only 2 of ~1,300 other sympatric rodents captured in the same surveys (Birtles, Bown and Telfer, unpublished observations). Although this suggests that field voles are the likely reservoir for *B. bennetti* sp. nov. in Kielder Forest, the infection prevalence at which it was detected was markedly lower than that for other co-circulating *Bartonella* species (18); thus, further work is required to clarify its ecology. The ecologies of '*B. bilalgolemii*' and AR15-3 are less certain as both were recovered from single individuals [20]. Nonetheless, the distinct host and geographical associations of these strains support the idea that ecological context, particularly host specificity, is an important criterion for resolving species boundaries in the *Bartonella* genus and that even highly similar genomes may represent distinct evolutionary lineages.

Phylogenetic placement supports host specificity over generalism

The phylogenetic placement of *B. bennetti* sp. nov. supports the view that host specificity, rather than generalism, may be the dominant ecological strategy within the L3 clade and broadly the *Bartonella* genus. A maximum-likelihood phylogeny of 52 publicly available *Bartonella* genomes, constructed using RAxML from 500 single-copy core genes, revealed a deep split between a basal lineage and a well-supported monophyletic clade of eubartonellae composed of 4 major lineages (Fig. 4). Within this framework, *B. bennetti* sp. nov. clustered within L3, alongside only three other characterized species: '*B. bilalgolemii*', *B. clarridgeiae* and *B. rochalimae*. This clade was highly resolved, with 100% bootstrap support across all nodes. Although *B. rochalimae* is frequently reported from a wide range of hosts, it is often identified primarily on the basis of partial *gltA* sequences [48], a low-resolution marker that lacks the discriminatory power to distinguish closely related but ecologically distinct strains. As such, the apparent host generalism of *B. rochalimae* may reflect methodological artefact rather than true ecological breadth. It remains plausible, and perhaps likely, that what is currently considered *B. rochalimae* in fact comprises a complex of cryptic, host-specific lineages [49]. In contrast, the other L3 members appear to show specific host associations: *B. clarridgeiae* with domestic cats (*Felis catus*) [16] and *B. bennetti* sp. nov. with field voles (*M. agrestis*). This pattern is consistent with ecological data indicating low host-switching rates in rodent-associated *Bartonella* species [21, 23, 50] and suggests that L3 species have evolved along narrow host-specialist trajectories.

Although the functional drivers of this pattern remain unclear, one explanation may lie in differences in host-adaptation machinery between lineages. Previous work has proposed that both L3 and L4 underwent parallel adaptive radiations, potentially triggered by the acquisition of the VirB/D4 T4SS and its associated effectors (Beps) [12]. However, L4 species, unlike those in L3, also encode the Trw T4SS, a system implicated in mediating host specificity and host switching, which is mutually exclusive with flagellar motility (Fig. 2). The absence of Trw in L3 taxa may limit their capacity for ecological diversification compared to L4. In contrast, some species within L4 have lost functionality in the ancestral Vbh system, which is retained in L2 and *B. australis* [14]. These patterns suggest a dynamic history of gain and loss in T4SS systems, which may have contributed to the differences in host range and ecological breadth observed between lineages. Together, the phylogenetic and ecological data indicate that *B. bennetti* sp. nov. and its closest relatives in L3 represent a lineage characterized more by host fidelity than flexibility, with the Trw system potentially facilitating broader host adaptation in L4 species rather than directly causing generalism.

DESCRIPTION OF *BARTONELLA BENNETTI* SP. NOV.

Bartonella bennetti (ben.net'ti. N.L. gen. n. *bennetti*, of Bennett, in honour of the veterinary microbiologist Malcolm Bennett, for his contributions to the fields of zoonoses and infectious disease ecology).

B. bennetti sp. nov. was isolated from a field vole (*M. agrestis*) in Kielder Forest, Northumberland, UK, in September 2004. The bacterium is a rod-shaped bacillus that measures 0.8–1.2 µm in length and 0.3–0.4 µm in width and possesses up to four polar lophotrichous flagella measuring up to 2 µm in length. *B. bennetti* sp. nov. forms round, small, white–yellow colonies on blood-enriched Columbia agar within 10 days when grown at 35–37 °C with 5% CO_2 or under microaerophilic, but not anaerobic, conditions. The bacterium is unable to reduce nitrates or produce indole and is unable to ferment glucose or utilize arginine or citrate. It does not have beta-galactosidase, catalase, gelatinase, oxidase or urease activity. It is unable to assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate or phenylacetic acid. The type strain C271^T contains a single circular chromosome (GenBank accession number CP174576.1) measuring 1.63 Mbp in length with a G+C content of 35.9 mol%. 16S rDNA data for C271^T is available under the accession number OR452865.1. C271^T has been deposited in the National Collection of Type Cultures, London, UK (NCTC15117), and in the Collection de Souches de l'Unité des Rickettsies, Marseille, France (CSUR B1113).

The genome of *B. heixiaziensis* strain RE21 is available under the accession CP176404.2 (RE21 chromosome and plasmid), and the strain has been deposited in the National Collection of Type Cultures, London, UK (NCTC15118), and in the Collection de Souches du Unite des Rickettsies, Marseille, France (CSUR B1114).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All research reported in this study was conducted in accordance with institutional and national guidelines and was approved by the University of Salford Ethics Committee.

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