Bartonella gabonensis sp. nov., a new bartonella species from savannah rodent Lophuromys sp. in Franceville, Gabon

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Abstract

We describe a new strain named Bartonella gabonensis sp. nov. strain 669^T (CSURB1083). The entire genome of this strain is described here. It was isolated from a savannah rodent, a brush-furred rat (Lophuromys sp.), trapped the city of Franceville in Gabon, in Central Africa. B. gabonensis is an aerobic, rod-shaped and Gram-negative bacterium. On the basis of the organism's features, and following a taxonogenomic approach, we propose the creation of the species Bartonella gabonensis sp. nov.

Keywords: *Bartonella gabonensis* sp. nov., Gabon, genome, *Lophuromys* sp., rodents
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Introduction

Bartonella is the only genus of the family Bartonellaceae within the Alphaproteobacteria class [1,2]. The members of this family are Gram negative, facultative intracellular and fastidious with slow growth. The genus Bartonella to date contains 37 known species and three subspecies (http://www.bacterio.net/bartonella.html). The majority of associations between Bartonella and their primary hosts are specific. The best-known example is Bartonella henselae, which has been found in domestic and wild Felidae worldwide, including Africa [3]. In addition, Bartonella species infect a wide range of hosts, including domestic animals as cats, dogs and cattle; wild animal such as bats, coyotes and foxes; and many rodent species.

The latest epidemiologic studies from around the world have shown a high prevalence and diversity of Bartonella species in rodents [4]. Several Bartonella species associated with rodent hosts have been involved in human pathologies, including endocarditis, myocarditis, fever, neurologic disorders, intraocular disorders, meningitis and splenomegaly [4]. Indeed, many studies have shown that rodents are important hosts of Bartonella spp. bacteria [1,5,6]. More recently, several new species of Bartonella have been isolated in rodents and their ectoparasites in sub-Saharan Africa, including Bartonella mastomydis [7], Bartonella massiliensis [8] and Bartonella saheliensis [9].

In Gabon, a previous study on rodent-borne infectious agents in the city of Franceville (Mangombi *et* al., unpublished data) identified three Bartonella species from rodent hosts: Bartonella elizabethae in Rattus rattus, Bartonella massiliensis in Cricetomys sp. and Candidatus *Bartonella gabonensis* in Lophuromys sp.

Here we aim to describe Bartonella gabonensis sp. nov. strains 662, 667 and 669, which we previously called Candidatus *Bartonella gabonensis* (Mangombi *et* al., unpublished data). This description also include the complete and annotated genome of this new species. Three strains were isolated from

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Lophuromys sp. rodents collected in the town of Franceville, Gabon.

Samples and bacterial culture

In Gabon, a Central African country, rodents were sampled in six districts and small savannah and forest islands within the city of Franceville in 2014 according to a standardized live-trapping protocol as previously described [10]. The districts sampled were Mbaya, Yéné, Sable, Mangoungou, Ombélé and Poto-poto (Potos). Mbaya and Yéné are the two main entry points to the city, by road or railway respectively. Sable and Mangoungou are more isolated districts. Mbaya is mainly industrial. Potos is the central trade district, including large storehouses and the main open market. Trapping campaigns were performed under the auspices of prior agreement from local authorities (city mayor and district chief), and all sampling procedures were approved by the Comité Nationale d'Ethique pour la Recherche (0020/2013/SG/CNE). Live-trapped rodents were brought back to laboratory, humanely killed with a halothane solution, necropsied according to a previously established protocol [11] and then weighed, sexed and measured. During necroscopy, various organs and tissues such as kidney, liver, brain, lungs and spleen were collected and stored at −80°C. However, only livers were used for the present study.

Total DNA was extracted from liver of rodent on the BioRobot EZ1 device (Qiagen, Courtaboeuf, France) using a commercial EZ1 DNA/RNA tissue kit (Qiagen) following the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed to screen all rodent samples for Bartonella sp. using the following primers: Barto ITS3, forward: GATGCCGGGGAAGGTTTTC, and reverse: ITS3 GCCTGGGAGGACTTGAACCT, and probe: Barto ITS3 P 6FAM-GCGCGCGCTTGATAAGCGTG. Conventional PCR was performed on all qPCR-positive samples using following primers: Urbarto1: 5' CTTCGTTTCTCTTTCTTCA 3' for forward; and reverse, Urbarto2: CTTCTCTTCA-CAATTTCAAT, as previously described [12,13].

From a total of 198 rodents tested by qPCR for Bartonella sp., only five rodents from two districts and an island savannah were infected, as follows: in Potos district, one (6.7%) of 15 B. elizabethae were infected; Sable district, one (4%) of 25 with B. massiliensis; and savannah, three (9.7%) of 34 with what is apparently a novel Bartonella genotype (Mangombi et al., unpublished data). Three strains were isolated from livers of three brush-furred rats of the genus Lophuromys (MT256381.1). Bartonella strains were cultured as previously reported [7,8,14].

Bartonella colonies were obtained from bacterial culture of liver of rodents previously found to be positive for Bartonella spp. by a Bartonella genus–specific qPCR after 5 to 10 days' incubation at 37°C. Culturing was performed in a 5% CO2-enriched atmosphere on Columbia agar plates supplemented with 5% sheep's blood (bioMérieux, Marcy l'Etoile, France) [3,8].

Classification and features

As previously described in similar studies [7,8], the internal transcribed spacer (ITS), gltA, rpoB, ftsZ and 16S ribosomal RNA (rRNA) genes were amplified and sequenced to identify isolated Bartonella strains. Three B. gabonensis isolates (strains 662, 667 and 669^T) were obtained from three individuals of the same rodent species, the rusty-bellied brush-furred rat Lophuromys sikapusi. We could not obtain all the sequences for the 662T strain. However, after analysis of the sequences obtained, the 667T and 669^T strains showed 100% identity between each other for the 16S rRNA gene; the other genes—in this case gltA, rpoB, ftsZ and ITS—showed 99% identity. This means that the three strains belong to the same species.

TABLE 1. Classification and general features of Bartonella gabonensis sp. nov. strain 669^T

MIGS ID	Property	Term	Evidence code[a]
	Current classification		
	Domain	Bacteria	TAS
	Phylum	*Proteobacteria*	TAS
	Class	*Alphaproteobacteria*	TAS
	Order	*Rhizobiales*	TAS
	Family	*Bartonellaceae*	TAS
	Genus	*Bartonella*	TAS
	Species	*Bartonella gabonensis*	IDA
	Type strain	669^T	IDA
	Gram stain	Negative	IDA
	Cell shape	Rod	IDA
	Motility	Nonmotile	IDA
	Sporulation	Nonsporulating	IDA
	Temperature range	Mesophilic	IDA
	Optimum temperature	37°C	IDA
MIGS-22	Oxygen requirement	Aerobic	IDA
	Carbon source	Unknown	IDA
	Energy source	Unknown	IDA
MIGS-6	Habitat	Rodents liver	IDA
MIGS-15	Biotic relationship	Facultative intracellular	IDA
	Pathogenicity	Unknown	IDA
	Biosafety level	3	IDA
MIGS-14	Isolation	*Lophuromys* sp.	IDA
MIGS-4	Geographic location	Franceville, Gabon	IDA
MIGS-5	Sample collection	14 April, 2014	IDA
MIGS-4.2	Latitude	1° 37' 59.9" S	IDA
MIGS-4.3	Longitude	13° 34' 59.9" E	IDA
MIGS-4.4	Altitude	372 m	IDA

MIGS, Minimum Information About a Genome Sequence.
[a]Evidence codes are as follows: IDA, inferred from direct assay; TAS, traceable author statement (i.e. a direct report exists in the literature). These evidence codes are from the Gene Ontology project (http://www.geneontology.org/GO.evidence.shtml). If the evidence code is IDA, then the property should have been directly observed, for the purpose of this specific publication, for a live isolate by one of the authors, or by an expert or reputable institution mentioned in the acknowledgements.

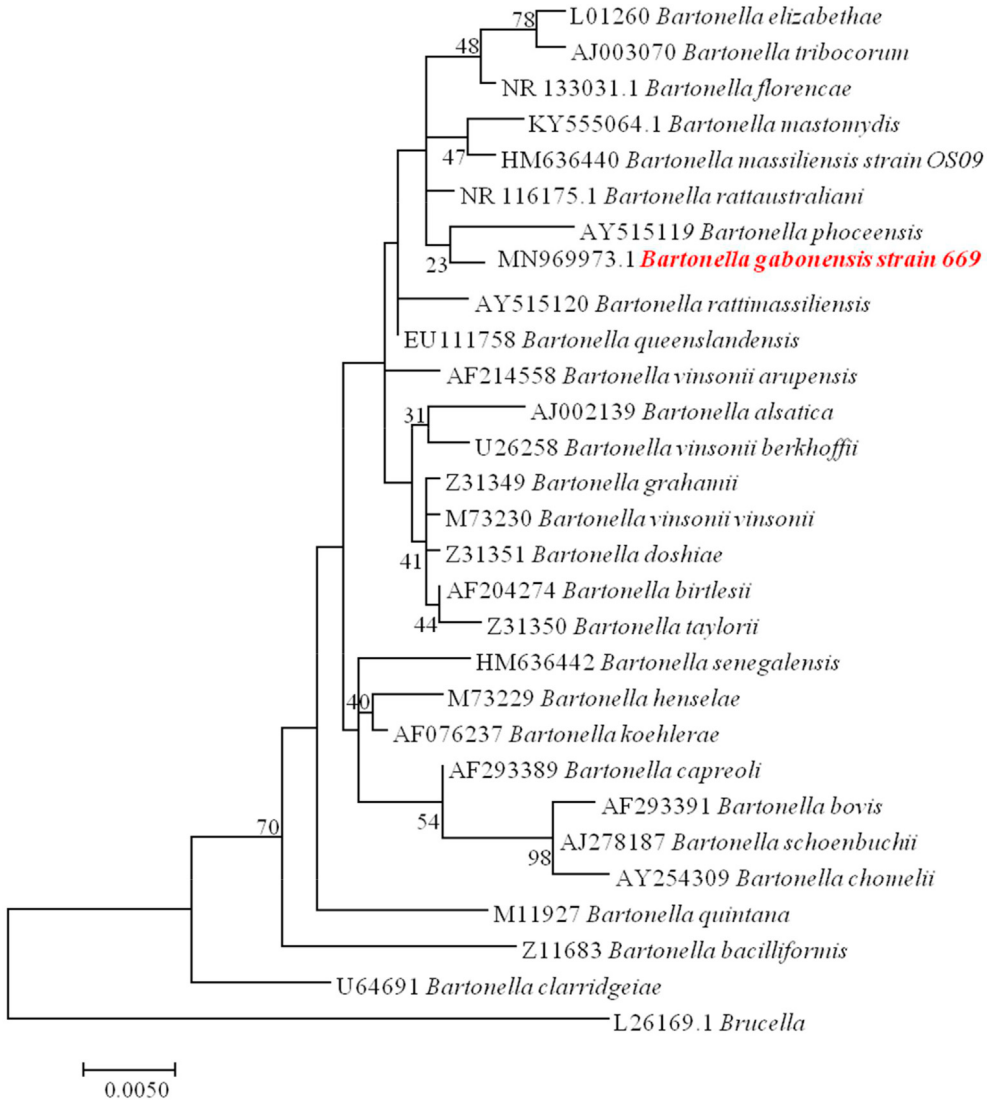

FIG. 1. Phylogenetic tree showing position of Bartonella gabonensis sp. nov. strain 669^T for 16S rRNA, compared with other phylogenetically close neighbours. Sequences were aligned using Clustal W parameters within MEGA 7 software. Evolutionary history was inferred using minimum evolution method. Respective GenBank accession numbers for 16S rRNA gene are indicated. Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 1000 times to generate majority consensus tree.

Here we describe strain 669^T (Table 1). Strain 669^T exhibited the closest identities in sequences with the different members of the Bartonella species as follows: for 16S rRNA gene, 99.43% with Bartonella grahamii (CP001562.1), for ITS gene 85.22% with Bartonella queenslandensis strain AUST/NH15 (EU111769.1), for gltA gene 94.14% with B. elizabethae strain NCTC12898 (LR134527.1), for rpoB gene 94.88% with Bartonella tribocorum strain ApoSilv-B29907 (JF766251.1) and for ftsZ gene 96.02% with B. elizabethae NCTC12898 (LR134527.1). According to La Scola *et al.* [15], these identity indices suggest that this strain may represent a new species within the Bartonella genus. The phylogenetic position of 669^T strain was illustrated by comparing it with other bacteria of the genus Bartonella using 16S rRNA sequences (Fig. 1).

Matrix-assisted desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) protein analysis was performed as previously described using a MicroFlex LT spectrometer (Bruker Daltonics, Bremen, Germany) [16]. The obtained spectra for 669^T strain were imported into MALDI Biotyper 3.0 software (Bruker) and analysed against the main spectra of bacteria, including spectra of the validly named Bartonella species, used as reference data in the BioTyper database (Fig. 2). No identification was possible with a score lower than 1.7. The 669^T strain had a score below 1.7 using

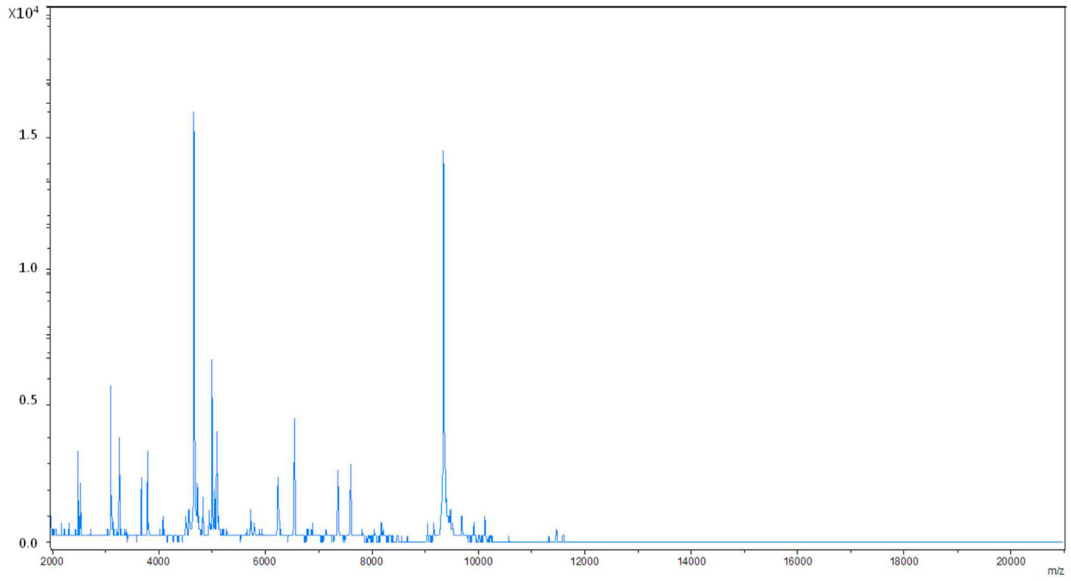

FIG. 2. Reference mass spectrum by MALDI-TOF MS analysis for Bartonella gabonensis strain 669^T. Spectra of some individual colonies (minimum >4) were compared and reference spectrum produced.

the IHU spectra database [17]. Therefore, no identification was possible. These data also suggest that isolate 669^T was not a member of a known species. A dendrogram made with Biotyper 3.0 software comparing the spectrum of the 669^T strain to those of the other Bartonella species is shown in Fig. 3.

Biochemical characterization

Different growth temperatures (32, 37 and 42°C) were tested. Optimal colony growth was observed at 37°C on Columbia agar supplemented with 5% sheep's blood in an atmosphere

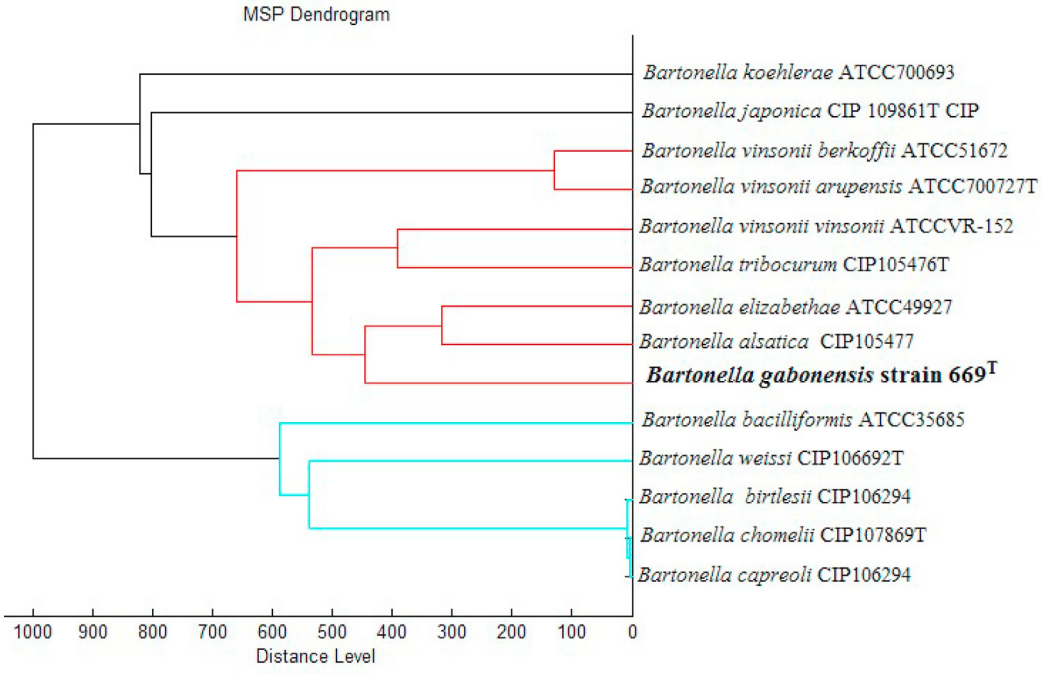

FIG. 3. Dendrogram comparing MALDI-TOF MS spectra of Bartonella gabonensis sp. nov. strain 669^T with those of some other members of Bartonella genus.

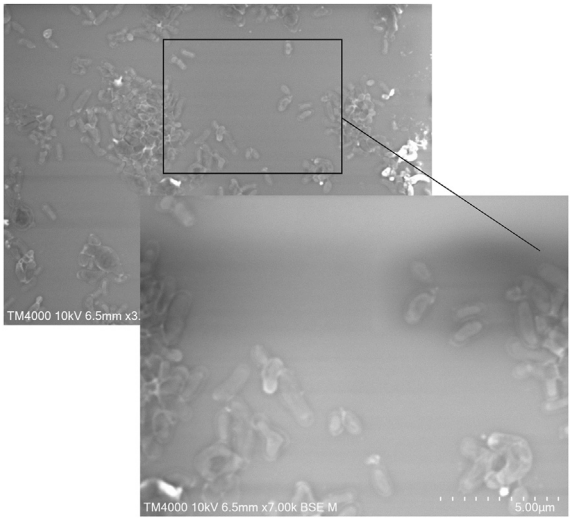

FIG. 4. Transmission electron micrograph of Bartonella gabonensis sp. nov. strain 669^T on TM4000Plus microscope (Hitachi High-Tech, Tokyo, Japan). Scale represents 5 µm.

enriched with 5% CO2. Colonies were grey and opaque with a diameter of 0.3 to 1 mm on Columbia sheep's blood–enriched agar. Bacterial cells were Gram negative and had a mean length and width of 1.36 ± 0.27 µm and 0.59 ± 0.11 µm respectively by electron microscopy (Fig. 4). Bartonella samples were fixed with a solution of glutaraldehyde 2.5% and cacodylate 1% in distilled water. Then 200 µL of fixed suspension composed to 100 µL of fixation solution and 100 µL of bacterial suspension in saline buffer (Thermo Fisher Scientific, Waltham, MA, USA) were centrifuged with a Thermo Scientific Cytospin centrifuge (Thermo Fisher) for 5 minutes at 254*g* (1500 rpm). After a contrast step with a solution of phosphotungstic acid hydrate, the slide was dried and observed under a TM4000Plus microscope (Hitachi High-Tech, Tokyo, Japan). Neither flagella nor pili were observed. Strain 669^T exhibited neither catalase nor oxidase activities. Biochemical characteristics were assessed using the following strips: API ZYM, 50 CH and API Coryne (bioMérieux). None of the available biochemical tests (catalase, oxydase, D-fructose, D-galactose, D-mannose) was positive. Similar patterns have been previously observed for Bartonella mastomydis [7], Bartonella massiliensis [8] and Bartonella saheliensis [9].

Genome sequencing information

Genome project history

Strain 669^T was selected for sequencing on the basis of the similarity of its 16S rRNA, ITS, ftsZ, gltA and rpoB sequences to other members of the genus Bartonella. Nucleotide sequence similarities for these genes suggested that strain 669 represents a new species in the genus Bartonella. Its genome was assembled and deposited under GenBank accession numbers CAHOYM010000001 to CAHOYM010000121. A summary of the project information is shown in Table 2.

TABLE 2. Project information

MIGS ID	Property	Term
MIGS-31	Finishing quality	High-quality draft
MIGS-28	Libraries used	One paired-end 3 kb library
MIGS-29	Sequencing platforms	454 GS FLX Titanium
MIGS-31.2	Fold coverage	28×
MIGS-30	Assemblers	gsAssembler from Roche
MIGS-32	Gene calling method	Prodigal
	GenBank ID	CAHOYM010000000
MIGS-13	Project relevance	Detection of *Bartonella* in rodent, *Lophuromys sp.*, from savannah of Gabon

MIGS, Minimum Information About a Genome Sequence.

Growth conditions and DNA isolation

Bacterial production for the genome sequencing was performed as follows. Bartonella gabonensis sp. nov. strain 669^T (CSURB1083) was cultured on Columbia agar enriched with sheep's blood (bioMérieux) in a 5% CO2 atmosphere at 37°C. Bacteria growing on three petri dishes were spread and resuspended in 3 × 100 µL of G2 buffer (EZ1 DNA Tissue kit; Qiagen). Genomic DNA of B. gabonensis sp. nov. strain 669^T was extracted in two steps. First, mechanical lysis was performed with glass powder using the Fastprep-24 device (MP Biomedicals, Graffenstaden, France) during 2 × 20 seconds. Then DNA was extracted via the EZ1 biorobot (Qiagen) with the EZ1 DNA tissue kit after 30 minutes' lysozyme incubation at 37°C. Genomic DNA was quantified by Quant-iT PicoGreen dsDNA assay kit (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) to 89.3 ng/µL.

Genome sequencing and assembly

Five micrograms of DNA was mechanically fragmented on a Hydroshear device (Digilab, Holliston, MA, USA) with an enrichment size of 3 to 4 kb. DNA fragments were visualized through an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) on a DNA LabChip 7500 device (Agilent) with an optimal size of 3.38 kb. The library was constructed according to the 454 GS FLX Titanium paired-end rapid library protocol. Circularization and nebulization were performed and generated an optimal pattern at 641 bp. After PCR amplification of almost 20 cycles, the double-stranded paired end library was then quantified on the Quant-iT RiboGreen kit (Invitrogen) on the Genios_Tecan fluorometer at 7360 pg/µL. The library concentration equivalence was

calculated as 1.14E+10 molecules/μL. The library was stored at −20°C until further use. The library was clonally amplified with 1.5 cpb in three-emulsion PCR (emPCR) reactions with the GS Titanium SV emPCR kit (Lib-L) v2 (Roche, Basel, Switzerland). The yield of the emPCR was 13.47%, which falls within the 5% to 20% range recommended in the Roche-specified procedures. A total of 790<thinsp>000 beads were loaded on a quarter region of the GS Titanium PicoTiterPlate PTP 70 × 75 kit and sequenced with the GS FLX Titanium Sequencing XLR70 kit (Roche). The run was analysed on the cluster through gsRunBrowser and gsAssembler (Roche). In total, 119<thinsp>842 passed filter wells were obtained and generated 38.01 Mb, with an average length of 317 bp. The passed filter sequences were assembled using gsAssembler with 90% identity and 40 bp as overlap. The final assembly identified 162 scaffolds and 121 large contigs (>1500 bp), which corresponds to 28 × as an equivalence genome.

Genome annotation

Prodigal was used for prediction in the open reading frame (ORF) with the default settings [18]. Deviations in the sequencing regions predicted by ORFs were excluded. BlastP

TABLE 3. Number of genes associated with general COGs functional categories

Code	Value	% of total[a]	Description
J	172	10.2	Translation
A	0	0	RNA processing and modification
K	57	3.4	Transcription
L	99	5.9	Replication, recombination and repair
B	0	0	Chromatin structure and dynamic
D	30	1.8	Cell cycle control, mitosis and meiosis
Y	0	0	Nuclear structure
V	34	2.0	Defense mechanisms
T	55	3.3	Signal transduction mechanisms
M	115	6.8	Cell wall/membrane biogenesis
N	6	0.4	Cell motility
Z	0	0	Cytoskeleton
W	3	0.2	Extracellular structures
U	60	3.6	Intracellular trafficking and secretion
O	80	4.8	Posttranslational modification, protein turnover, chaperones
X	36	2.1	Mobilome: prophages, transposons
C	85	5.1	Energy production and conversion
G	61	3.6	Carbohydrate transport and metabolism
E	116	6.9	Amino acid transport and metabolism
F	49	2.9	Nucleotide transport and metabolism
H	74	4.4	Coenzyme transport and metabolism
I	46	2.7	Lipid transport and metabolism
P	65	3.9	Inorganic ion transport and metabolism
Q	14	0.8	Secondary metabolites biosynthesis, transport and catabolism
R	98	5.8	General function prediction only
S	85	5.1	Function unknown
—	442	26.3	Not in COGs

COGs, Clusters of Orthologous Groups database.
[a]Total is based on total number of protein-coding genes in annotated genome.

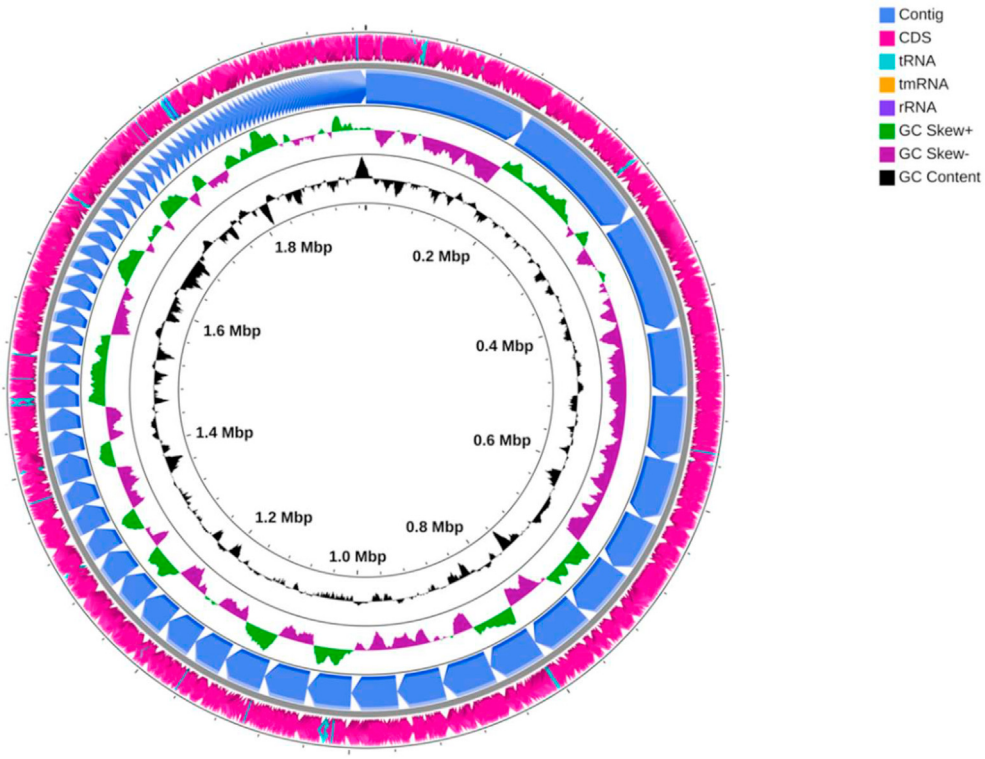

FIG. 5. Circular map generated by CGView Server (https://paulstothard.github.io/cgview/) showing complete view of genome of Bartonella gabonensis sp. nov.

TABLE 4. Numerical DNA-DNA hybridization values (%) obtained by pairwise comparison between Bartonella gabonensis sp. nov. and other closely related species using GGDC formula 2 software (DDH estimates based on HSP identities/length)

	B. gabonensis	*B. ancashensis*	*B. australis*	*B. bacilliformis*	*B. elizabethae*	*B. henselae*	*B. quintana*	*B. rattimassiliensis*	*B. tribocorum*	*B. vinsonii*
B. gabonensis	100	15.4% ± 3.2	17.1% ± 3.3	19% ± 3.3	80% ± 3.7	38.6% ± 3.4	37.9% ± 3.4	54.3% ± 3.5	65.% ± 3.75	41.3% ± 3.4
B. ancashensis		100	15.4% ± 3.2	22% ± 3.4	15.2% ± 3.1	16.2% ± 3.2	16.1% ± 3.2	15.1% ± 3.15	14.7% ± 3.15	16.8% ± 3.25
B. australis			100	20% ± 4	16.6% ± 3.2	17.% ± 3.3	19.8% ± 3.4	15.4% ± 3.2	15.% ± 3.2	17.8% ± 3.3
B. bacilliformis				100	20.1% ± 3.4	21.9% ± 3.4	25% ± 3.5	19% ± 3.4	18.4% ± 3.35	23.7% ± 3.5
B. elizabethae					100	39.1% ± 3.45	38.1% ± 3.4	53.9% ± 3.5	64.8% ± 3.7	39.7% ± 3.45
B. henselae						100	62.1% ± 3.65	34.3% ± 3.45	35.8% ± 3.45	49.3% ± 3.45
B. quintana							100	33.2% ± 3.45	31.8% ± 3.45	54% ± 3.5
B. rattimassiliensis								100	58.8% ± 3.6	35.8% ± 3.45
B. tribocorum									100	36.2% ± 3.45
B. vinsonii										100

was used to predict the bacterial proteome (*E* value of 1e-03, coverage of 0.7 and percentage identity of 30) according to the Clusters of Orthologous Groups (COGs) database. If there was no match, the BlastP database search [19] was extended with an *E* value of 1e-03, coverage of 0.7 and percentage identity of 30. However, if the length of the sequence was less than 80 aa, then an *E* value of 1e-05 was used. The 'hmmscan' analysis tool was used in the domains that are maintained by the Pfam database (Pfam-A and Pfam-B domains). The rRNA and transfer RNA (tRNA) genes were retrieved using the RNAmmer [20] and tRNAScanSE [21] tools. ORFans were identified when the BlastP *E* value was less than 1e-03 for an alignment length of >80 aa. We also used the online Genome-to-Genome Distance Calculator tool (https://ggdc.dsmz.de/ggdc.php#) to calculate DNA:digital DNA hybridization estimates (dDDH) with confidence intervals according to recommended parameters (formula 2, BLAST; Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The pan-genome distribution of B.

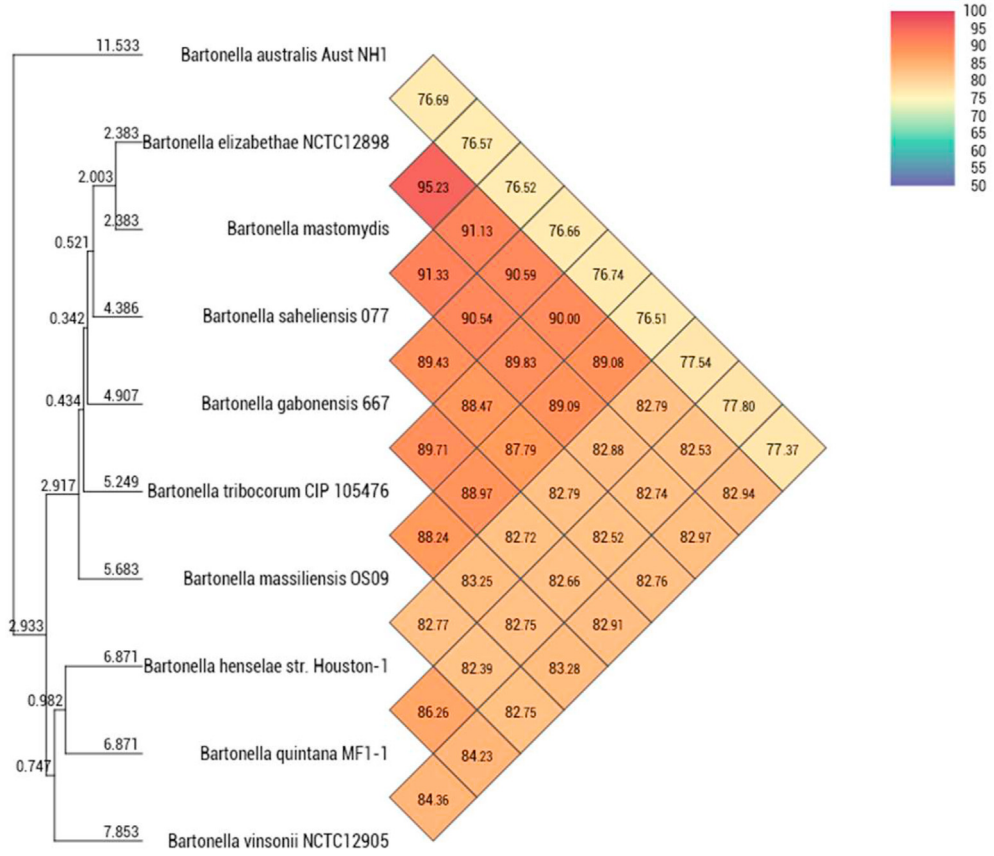

FIG. 6. Heat map generated using OrthoANI values calculated using OAT software between Bartonella gabonensis sp. nov. strain 669^T and other closely related species.

gabonensis and other closely related species was evaluated by Raory software [22].

Genome properties

The genome of B. gabonensis was 1,971,183 bp long with a 41.1 mol% G + C content [23] (Fig. 5). Of the 1736 predicted genes, 1680 were protein-coding genes and four were rRNAs, 11 were misc_RNA, 40 were tRNA and one was transfer-messenger RNA. The distribution of genes into COGs functional categories is presented in Table 3. The dDDH values obtained from B. gabonensis compared with other close strains are presented in Table 4. These dDDH values were below 70% of the recommended threshold for species demarcation [24]. Using dDDH analysis, values ranged from 20.00% between B. gabonensis and B. australis Aust/NH1, to 58.90% between B. mastomydis and B. elizabethae NCTC12898. Genes with a putative function (by COGs) numbered 1440 for B. gabonensis (76%). Finally, 442 genes (24%) were annotated as hypothetical proteins for strain B. gabonensis (Fig. 3).

The degree of genomic similarity of B. gabonensis with closely related species was estimated by OrthoANI software [25]. Finally, OrthoANI analysis showed that the higher percentage value was 95.23% between B. elizabethae NCTC12898 and B. mastomydis, while the lowest was 76.51% between B. australis Aust/NH1 and B. massiliensis. In addition, when B. gabonensis was compared with other species, the values ranged from to 76.66% with B. australis Aust/NH1 to 90.59% with B. elizabethae NCTC12898 (Fig. 6).

Conclusion

On the basis of phylogenetic, phenotypic and genomic analyses, as well as sequencing of the 16S rRNA, ITS, ftsZ, rpoB and gltA genes and MALDI-TOF MS spectra, we propose strain 669^T as the type strain of Bartonella gabonensis sp. nov., an undoubtedly new species of the Bartonella genus within the family Bartonellaceae. Strain 669 was isolated from the savannah rodent Lophuromys sp., trapped in the city of Franceville, in the south-west of Gabon in Central Africa.

Description of Bartonella gabonensis sp. nov

Bartonella gabonensis sp. nov. (ga.bo.nen'se, L. masc. adj. gabonensis, referring to Gabon, the Central African country where the rodent from which the type strain was isolated comes from). Optimal growth is observed at 37°C in an aerobic atmosphere. Colonies are grey and opaque, with a diameter of 0.3 to 1 mm on Columbia sheep's blood–enriched agar. Bacterial cells were Gram negative, and length and width were 1.36 ± 0.27 µm and 0.59 ± 0.11 µm respectively. Cells are rod shaped without flagella or pili. Bartonella gabonensis sp. nov. strain 669^T exhibits few biochemical and enzymatic activities.

Nucleotide sequence accession number

The 16S rRNA, ITS, ftsZ, rpoB and gltA gene sequences and genome sequences of Bartonella gabonensis sp. nov. strain 669^T are deposited in GenBank under accession numbers MN969973.1, MT003981, MT003982, MT274297, MT274298, MT274299, MT274300, MT274301, MT274302 and CAHOYM010000000 respectively.

Deposit in culture collections

Strain 669^T was deposited in strain collections under accession number CSURB1083.

Conflict of Interest

None declared.

Acknowledgements

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