Bartonella massiliensis sp. nov., a new bacterial species isolated from an *Ornithodoros sonrai* tick from Senegal

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Abstract

Bartonella massiliensis sp. nov., strain OS09^T (= CSURB624^T = DSM 23169), is the type strain of *Bartonella massiliensis* sp. nov., a new species within the genus *Bartonella.* It was isolated from a soft tick, *Ornithodoros sonrai,* vector of recurrent fever collected from Senegalese domestic rodent burrows. This strain is an aerobic, rod-shaped and Gram-negative bacterium. On the basis of taxonogenomic approach, we propose the creation of *Bartonella massiliensis* sp. nov.

Keywords: *Bartonella massiliensis*sp. nov., genome, *Ornithodoros sonrai*, senegal, soft tick
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Introduction

Bartonella is the monotypic genus of the family *Bartonellaceae* among *Alphaproteobacteria* [1]. *Bartonella* species are fastidious Gram-negative, slightly curved rod bacteria characterized by a small cell size (0.5–0.6 × 1.0 μm) [2]. They are facultative intracellular bacteria with a unique intraerythrocyte lifestyle. Currently the *Bartonella* genus includes 35 validly published species and three subspecies [3,4]. *Bartonella* species usually colonize the intestine of the arthropod vector or the bloodstream of the mammalian host [4,5]. In addition, our understanding of the involvement of these microorganisms in human diseases continues to grow, as does the range of clinical manifestations [6,7]. At least 13 *Bartonella* species are responsible for human diseases, including *B. bacilliformis*, *B. quintana* and *B. henselae,* which cause Carrión disease, trench fever and cat-scratch disease respectively. *Bartonella* species are also associated with chronic bacteraemia and/or endocarditis, bacillary angiomatosis, peliosis hepatis, prolonged fever of unknown origin, retinitis, uveitis and myocarditis in humans [6]. Other mammalian species that may host *Bartonella* species include dogs, coyotes, foxes, cattle, deer, elk, bats and many rodent species [8–10].

Here we present the description of *Bartonella massiliensis* strain OS09^T (= CSURB624^T = DSM 23169), a new species of the genus *Bartonella* isolated from a soft tick, *Ornithodoros sonrai,* including its complete annotated genome.

Samples and bacterial culture

Between September 2008 and May 2009, a research study on *Bartonella* species in *Ornithodoros sonrai,* a soft tick collected in Senegal (West Africa), was conducted by Mediannikov et al. [11]. Sampling was carried out in populated houses with numerous rodent burrows in room floors. Morphologically, all ticks collected in domestic rodent burrows have been identified as *Ornithodoros sonrai,* a nidicolous tick that inhabits small mammal burrows [11]. Globally, ticks from only two of the villages (Soulkhou Thissé and Maka Gouye) were infected with *Bartonella* spp., with infection in 62.5% (5/8) of 4.2% (1/24) respectively. Sequences of internal transcribed spacer (ITS) amplicons obtained from these ticks showed that the *Bartonella* identified in ticks collected in the two villages differed

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from each other insignificantly (0.3–3%), as well as from any other validly described species with standing in nomenclature (http://www.bacterio.net/bartonella.html). Culture of *Bartonella* strains was carried out as previously reported [11]. Briefly, the bacterial colonies of strains retrieved from *O. sonrai* were obtained after 5 to 7 days' incubation at 37°C in a 5% CO_2-enriched atmosphere on Columbia agar plates supplemented with 5% sheep's blood (bioMérieux, Marcy l'Etoile, France).

Classification and features

The ITS, *ftsZ*, *rpoB* and *gltA* genes as well as the 16S ribosomal RNA (rRNA) gene were amplified and sequenced to identify isolated *Bartonella* strains. After the sequences analysis, two strains, OS09^T and OS23^T, showed almost the same genetic similarity: they had 100% identity for the 16S rRNA and *rpoB* genes, 99.8% for the *ftsZ* gene and 99.9% for the *gltA* gene. No mutation was detected for the ITS gene, and only a 5 bp deletion was found for the OS23^T strain. The similarities of the sequences of the OS09^T and OS23^T strains with respect to the different species closest to the genus *Bartonella* for the 16S rRNA, ITS, *ftsZ*, *rpoB* and *gltA* genes were 99.5%, 79.5–81.3%, 96.8–96.9%, 93.2% and 94.5–94.6% respectively. According to La Scola et al. [8], these data support the notion that strains OS09^T and OS23^T may be identified as a new and same species. We focus here on the analysis of *Bartonella massiliensis* strain OS09^T (Table 1). Similarities in sequences of the OS09^T strain with the different closest members of the *Bartonella* species for the 16S rRNA gene, ITS, *ftsZ*, *rpoB* and *gltA* were 99.5%, 79.5%, 96.6%, 93.2% and 94.5%, with *Bartonella queenslandensis* (EU111758), *Bartonella elizabethae* (JF766264), *Bartonella grahamii* (CP001562), *Bartonella tribocorum* (JF766251) and *Bartonella grahamii* (CP001562) respectively. All 16S rRNA sequences of *Bartonella* species are used in Fig. 1 to highlight the phylogenetic position of this bacterium relative to other species.

MALDI-TOF MS was performed on a Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany) as previously described [9]. The obtained spectra (Fig. 2) were imported into MALDI Biotyper 3.0 software (Bruker) and analysed against the main spectra of bacteria included in two databases (Bruker as well as Microbes Evolution Phylogeny and Infections (MEPHI), which is constantly updated). No identification was obtained because the strain displayed scores below 1.7, supporting the suggestion that our isolate was not a member of a known species. The spectrum of strain OS09^T has been added to the local MEPHI database. A dendrogram made with Biotyper 3.0 software comparing the spectrum of the OS09 strain to those of the other *Bartonella* species is shown in Fig. 3.

TABLE 1. Classification and general features of *Bartonella massiliensis* sp. nov., strain OS09^T

MIGS ID	Property	Term	Evidence code[a]
	Current classification	Domain: *Bacteria*	TAS [12]
		Phylum: *Proteobacteria*	TAS [13,14]
		Class: *Alphaproteobacteria*	TAS [15]
		Order: *Rhizobiales*	TAS [16,17]
		Family: *Bartonellaceae*	TAS [18,19]
		Genus: *Bartonella*	TAS [18,20–22]
		Species: *Bartonella massiliensis*	IDA
		Type strain: OS09^T	IDA
	Gram stain	Negative	IDA
	Cell shape	Rod	IDA
	Motility	Nonmotile	IDA
	Sporulation	Nonsporulating	IDA
	Temperature range	Mesophilic	IDA
	Optimum temperature	32°C	IDA
MIGS-22	Oxygen requirement	Aerobic	IDA
	Carbon source	Unknown	IDA
	Energy source	Unknown	IDA
MIGS-6	Habitat	Tick gut	IDA
MIGS-15	Biotic relationship	Facultative intracellular	IDA
	Pathogenicity	Unknown	IDA
	Biosafety level	3	IDA
MIGS-14	Isolation	*Ornithodoros sonrai*	IDA
MIGS-4	Geographic location	Senegal	IDA
MIGS-5	Sample collection	May 2009	IDA
MIGS-4.1	Latitude	14°03′N	IDA
MIGS-4.2	Longitude	15°31′W	IDA
MIGS-4.3	Depth	~0.5 m under surface	IDA
MIGS-4.4	Altitude	5 m above sea level	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) [23]. 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.

Biochemical characterization

Different growth temperatures (32, 37 and 42°C) were tested. Optimal colony growth was observed at 32°C on Columbia agar supplemented with 5% sheep's blood in an atmosphere enriched with 5% CO_2. Colonies appeared grey and opaque, with a diameter of 0.3 to 1 mm on Columbia blood-enriched agar. The bacterial cells were Gram negative and had a mean length of 1.34 ± 0.26 µm and a width of 0.49 ± 0.13 µm. Neither flagella nor pili were observed by electron microscopy (Fig. 4). Strain OS09^T exhibited no catalase or oxidase activity. Biochemical characteristics were assessed by API strips ZYM, 50 CH and Coryne (bioMérieux). None of the available biochemical tests was positive. Similar patterns have been previously observed for *Bartonella senegalensis* and *Bartonella mastomydis* [10,24].

Genome sequencing information

Genome project history. The OS09 strain was selected for sequencing on the basis of its phylogenetic position and phenotypic differences with other members of the *Bartonellaceae* family. This strain was isolated in a study on the role of the soft tick, *O. sonrai,* as a host of *Bartonella* [11]. Currently 29

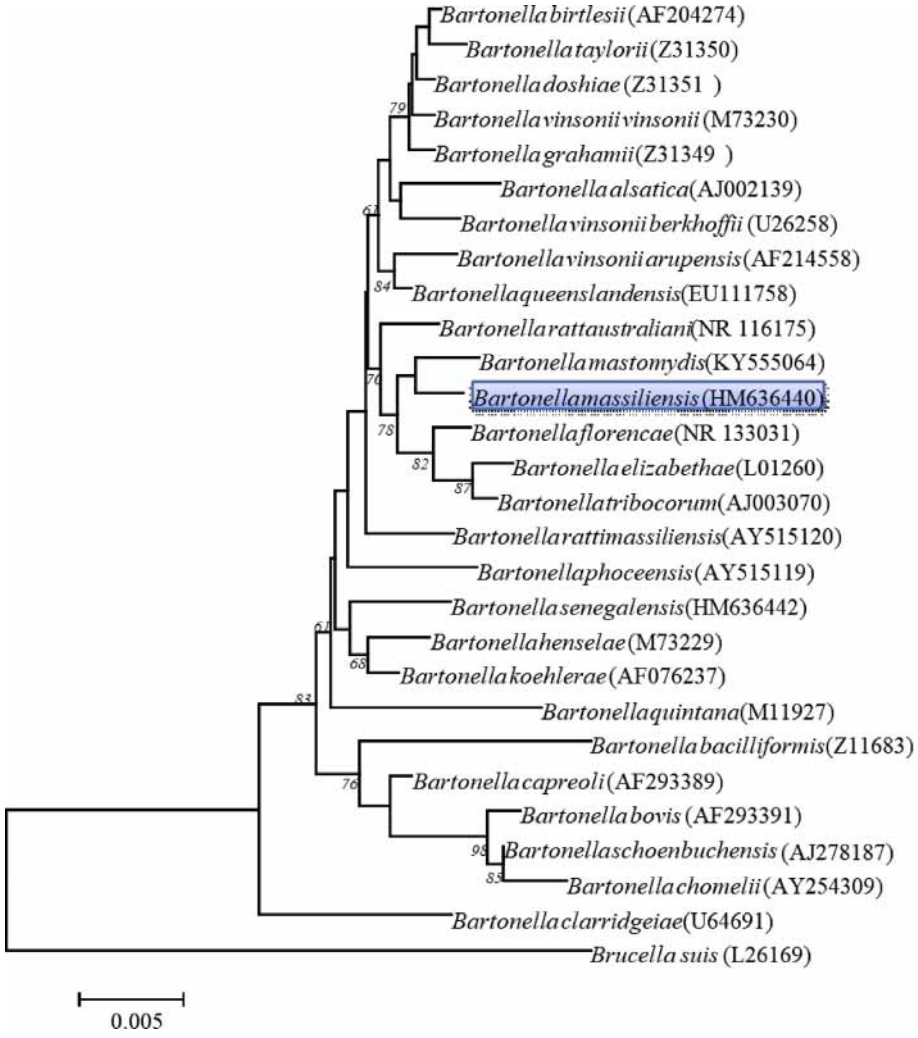

FIG. 1. Phylogenetic tree showing position of *Bartonella massiliensis* sp. nov., strain OS09^T, Relative to other phylogenetically close neighbours. Sequences were aligned by ClustalW parameters within MEGA7 software. Evolutionary history was inferred using minimum evolution method. Respective GenBank accession numbers for 16S rRNA genes are indicated in parentheses. Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 1000 times to generate majority consensus tree. Scale bar indicates 5% nucleotide sequence divergence.

genomes are available in GenBank database for the genus *Bartonella*. The genome of strain OS09^T is the first genome of *Bartonella massiliensis* sp. nov., and is assembled and deposited under GenBank accession numbers CABFVS010000001 to CABFVS010000091. A summary of the project information is presented in Table 2.

Growth conditions and DNA isolation. The OS09^T strain of *Bartonella massiliensis* (= CSUR B624T = DSM 23169) was cultured on Columbia agar enriched with sheep's blood (bioMérieux) with 5% CO_2 at 32°C. Bacteria growing on two petri dishes were harvasted and resuspended in 6 × 100 µL of G2 buffer. A first mechanical lysis was performed with glass powder using the Fastprep-24 device (MP Biomedicals, Graffenstaden, France) during 2 × 20 seconds. Then after 30 minutes' lysozyme incubation at 37°C, DNA was extracted on the EZ1 biorobot (Qiagen, Hilden, Germany) with the EZ1 DNA tissue kit. DNA was quantified by Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) to 68.6 ng/µL.

Genome sequencing and assembly. Five micrograms of DNA was fragmented mechanically on the Hydroshear device (Digilab, Holliston, MA, USA) with an enrichment size of 3 to 4 kb. The DNA fragments were visualized through an Agilent 2100 Bio-Analyzer (Agilent Technologies, Santa Clara, CA, USA) on a DNA lab chip 7500 with an optimal size of 3.75 kb. The library was constructed according to the 454_Titanium paired end rapid library protocol and the manufacturer. Circularization and nebulization were performed and generated a pattern optimal at 591 bp. After PCR amplification through 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

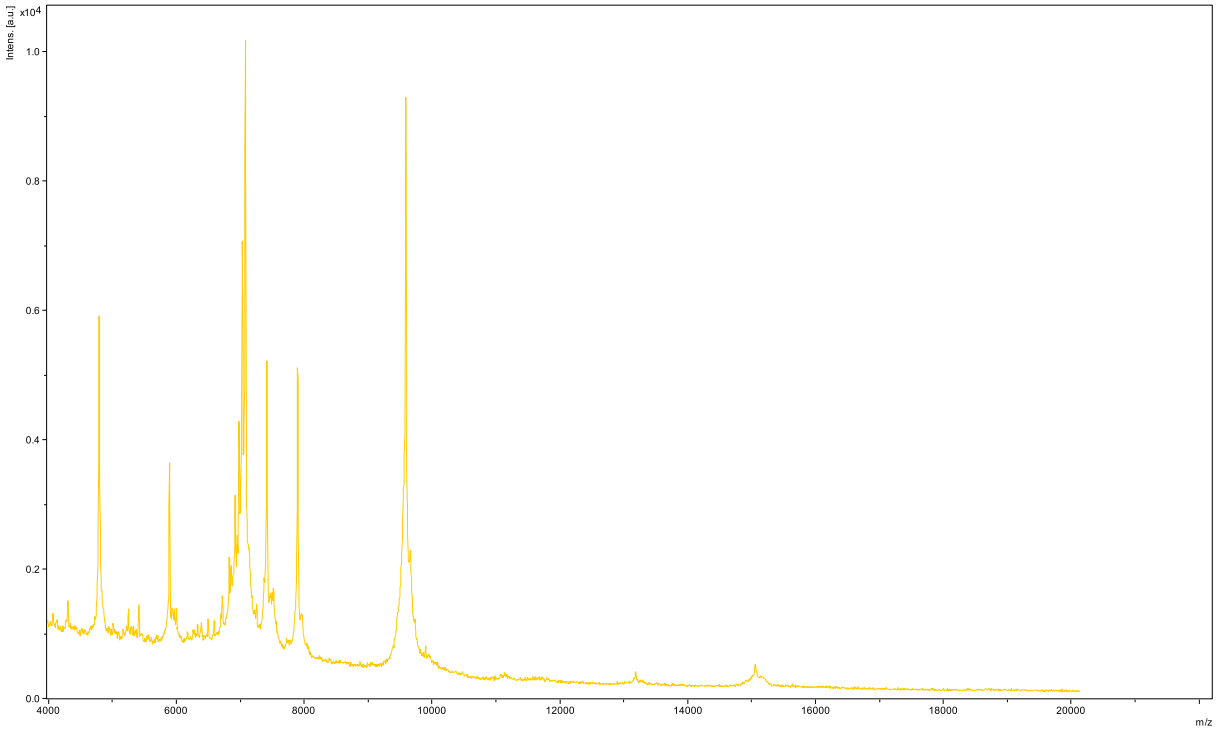

FIG. 2. MALDI-TOF MS reference mass spectrum of *Bartonella massiliensis* sp. nov. Spectra from 12 individual colonies were compared and reference spectrum generated.

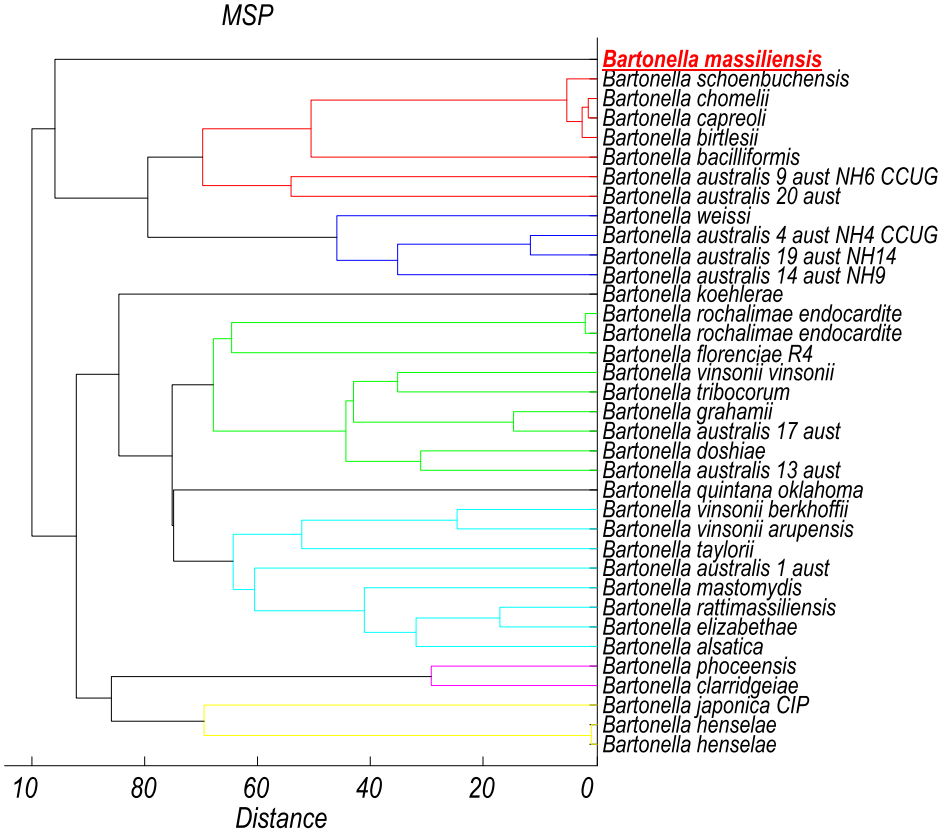

FIG. 3. Dendrogram comparing MALDI-TOF MS spectra of *Bartonella massiliensis* sp. nov., strain OS09^T, with those of other members of *Bartonella* genus.

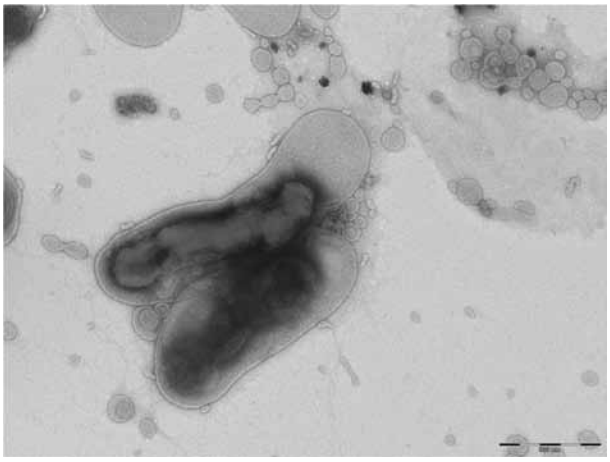

FIG. 4. Transmission electron micrograph of *Bartonella massiliensis* strain OS09^T using Morgagni 268D (Philips, Amsterdam, The Netherlands) transmission electron microscope at operating voltage of 60 kV. Scale bar represents 500 nm.

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	17.27×
MIGS-30	Assemblers	gsAssembler from Roche
MIGS-32	Gene calling method	Prodigal
	GenBank ID	CABFVS010000001-CABFVS010000091
MIGS-13	Project relevance	Detection of *Bartonella* in soft ticks, *Ornithodoros sonrai*

MIGS, Minimum Information About a Genome Sequence.

equivalence was calculated as 1.14E + 10 mol/µL. The library was stored at −20°C until use. The library was clonal amplified with 0.40 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%, within the range of 5% to 20% from the Roche procedure. A total of 790 000 beads were loaded on a quarter region of the GS Titanium PicoTiterPlate PTP Kit 70x75 and sequenced with the GS Titanium Sequencing Kit XLR70. The run was performed overnight, then analysed on the cluster through gsRunBrowser and gsAssembler (Roche). Overall, 119 842 passed filter wells were obtained and generated 38.01 Mb with an average length of 317 bp. The passed filter sequences were assembled on the gsAssembler with 90% identity and 40 bp as overlap. It led to 25 scaffolds and 234 large contigs (>1500 bp) and generated a genome size of 2.05 Mb, which corresponds to a coverage of 17.27× genome equivalent.

TABLE 3. Nucleotide content and gene count levels of genome

	Genome (total)	
Attribute	**Value**	**% of total[a]**
Genome size (bp)	2 277 694	100
G+C content (bp)	860 116	37.76
Coding region (bp)	1 653 384	72.59
Total genes	1967	100
RNA genes	42	2.14
Protein-coding genes	1925	97.86
Genes with function prediction	1309	68
Genes assigned to COGs	1362	70.75
Genes with signal peptides	252	13.09
Genes with transmembrane helices	388	20.16

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

Genome annotation

Open reading frames (ORFs) were predicted using Prodigal [25] with default parameters, but predicted ORFs were excluded if they spanned a sequencing gap region. The predicted bacterial protein sequences were searched against the GenBank database using BLASTP and the Clusters of Orthologous Groups (COGs) database using Cognitor [26]. The prediction of RNA genes, including rRNAs, transfer RNAs and other RNAs, was carried out using the RNAmmer [27] and ARAGORN [28] algorithms. The transmembrane helices and signal peptides were identified by TMHMM [29] and SignalP [30] respectively.

Genome properties

The genome is 2 227 694 bp long with 37.76 mol% GC content (Table 3, Fig. 5). It is composed of 91 contigs. Of the 1967 predicted genes, 1925 were protein-coding genes and 42 were RNAs (including one 16S rRNA, one 23S rRNA, one 5S rRNA and 39 transfer RNA genes). A total of 1309 genes (68%) were assigned a putative function (by COGs or NR BLAST). A total of 111 genes were identified as ORFans (5.77%). The remaining genes (n = 386) were annotated as hypothetical proteins (20.05%). The distribution of genes into COGs functional categories is presented in Table 4. The properties and statistical information of the genome are summarized in Tables 3 and 4. The degree of genomic similarity of OS09^T closely related species was estimated by OrthoANI software [31]. Values among closely related species ranged from 81.45% between *Bartonella massiliensis* strain OS09^T and *Bartonella rattaustraliani* AUST NH4 to 91.49% between *Bartonella queenslandensis* strain AUST NH15 and *Bartonella tribocorum* strain CIP 105476 (Fig. 6). When the isolate was compared to these closely species, values ranged from 81.45% with *Bartonella rattaustraliani* AUST NH4 to 89.09% with *Bartonella mastomydis* strain 008.

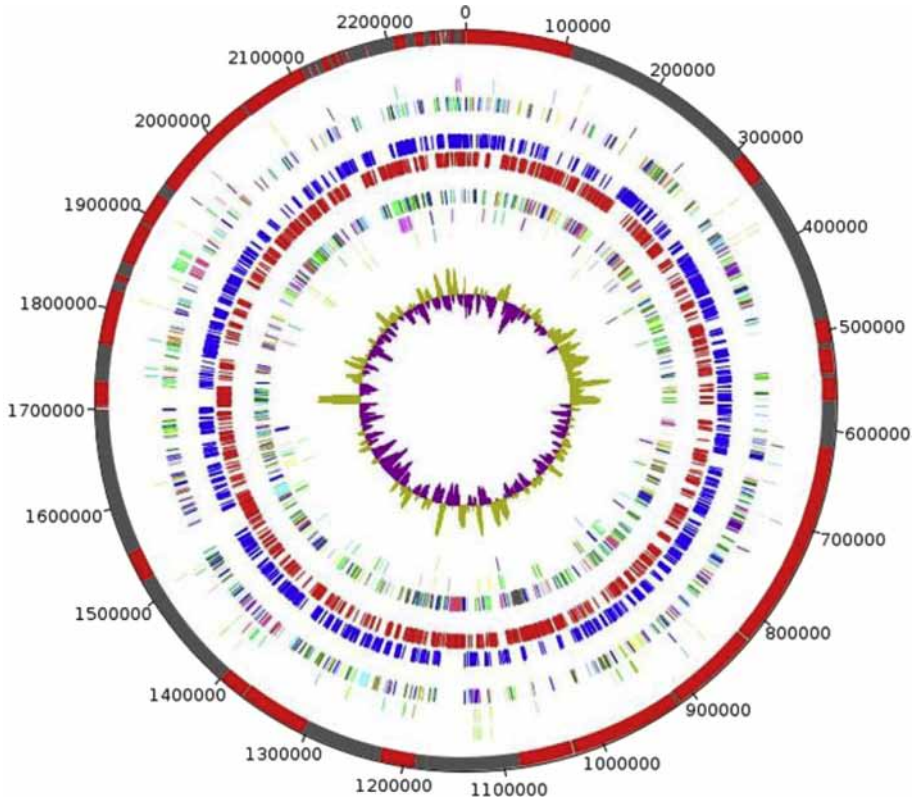

FIG. 5. Graphical circular map of chromosome. From outside to centre: genes on forward strand coloured by Clusters of Orthologous Groups database (COGs) categories (only genes assigned to COGs), genes on reverse strand coloured by COGs categories (only gene assigned to COGs), RNA genes (transfer RNAs green, rRNAs red), GC content and GC skew (three circles), GC content.

TABLE 4. Number of genes associated with 25 general COGs functional categories

Code	Value	% of total[a]	Description
J	146	7.58	Translation
A	0	0	RNA processing and modification
K	86	4.47	Transcription
L	128	6.65	Replication, recombination and repair
B	0	0	Chromatin structure and dynamic
D	24	1.25	Cell cycle control, mitosis and meiosis
Y	0	0	Nuclear structure
V	12	0.62	Defense mechanisms
T	44	2.29	Signal transduction mechanisms
M	100	5.19	Cell wall/membrane biogenesis
N	8	0.42	Cell motility
Z	1	0.05	Cytoskeleton
W	12	0.62	Extracellular structures
U	80	4.16	Intracellular trafficking and secretion
O	73	3.79	Posttranslational modification, protein turnover, chaperones
C	79	4.10	Energy production and conversion
G	73	3.79	Carbohydrate transport and metabolism
E	130	6.75	Amino acid transport and metabolism
F	47	2.44	Nucleotide transport and metabolism
H	58	3.01	Coenzyme transport and metabolism
I	41	2.13	Lipid transport and metabolism
P	84	4.36	Inorganic ion transport and metabolism
Q	15	0.78	Secondary metabolites biosynthesis, transport and catabolism
R	209	10.86	General function prediction only
S	119	6.18	Function unknown
—	563	29.25	Not in COGs

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

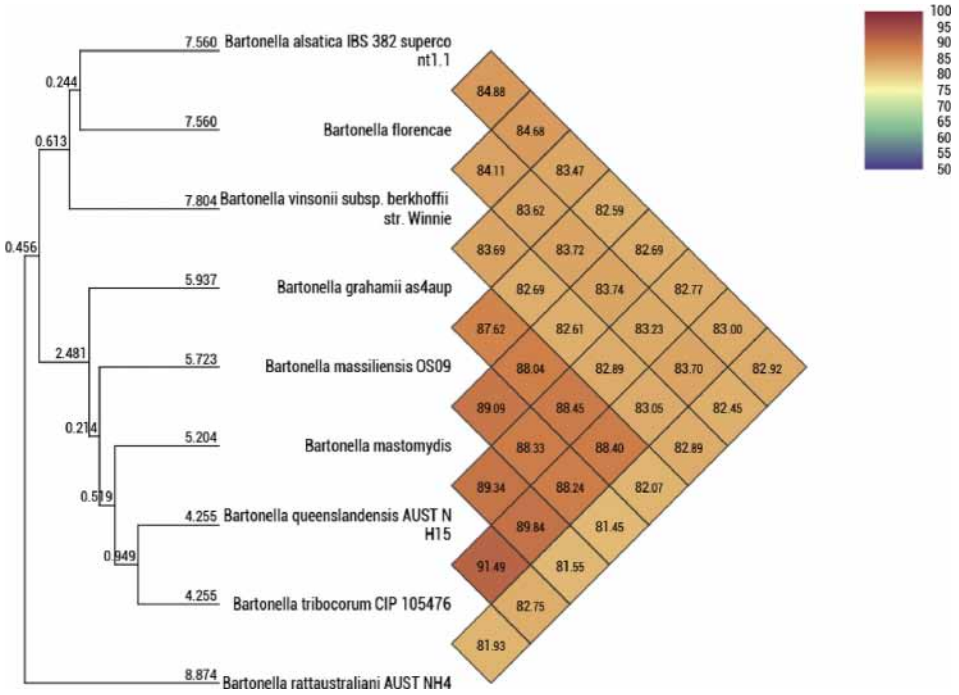

FIG. 6. Heat map generated with OrthoANI values calculated by OAT software between *Bartonella massiliensis* sp. nov., strain OS09^T and other closely related species with standing in nomenclature.

Conclusion

On the basis of unique phenotypic and genotypic characteristics, including MALDI-TOF MS spectrum, sequencing of the 16S rRNA, ITS, *ftsZ*, *rpoB* and *gltA* genes (sequence divergences >99.5%, >79.5%, >96.6%, >93.2% and >94.5% respectively) and an OrthoANI value lower than 95% with the phylogenetically closest species with standing in nomenclature, we consequently propose strain OS09^T as the type strain of *Bartonella massiliensis* sp. nov., a new bacterial species within the family *Bartonellacae*. The strain was isolated from rodent ticks, *O. sonrai,* collected in rural areas of Senegal (West Africa).

Description of *Bartonella massiliensis* sp. nov.

Bartonella massiliensis sp. nov. (mas.si.li.en'sis, L. masc. adj. *massiliensis,* 'of Massilia,' the ancient Roman name of Marseille, where the strain was isolated) is a nonmotile, Gram-negative rod. Optimal growth is observed at 32°C in an aerobic atmosphere. Colonies are opaque and grey, with a diameter of 0.3 to 1 mm on Columbia blood-enriched agar. Length and width are 1.34 ± 0.26 µm and 0.49 ± 0.13 µm respectively. Cells are rod shaped without flagella or pili. *Bartonella massiliensis* sp. nov., strain OS09^T exhibits neither biochemical nor enzymatic activities. The genome size and GC content are 2.22 Mb and 37.76 mol% respectively. The type strain OS09^T (= CSUR B624T = DSM 23169^T) was isolated from the rodent tick, *Ornithodoros sonrai,* collected in a rural area named Maka Gouye (14°03′N, 15°31′W) located in Senegal.

Nucleotide sequence accession number

The 16S rRNA, ITS, *ftsZ*, *rpoB* and *gltA* gene sequences and genome sequences of *Bartonella massiliensis* sp. nov., strain OS09^T, are deposited in GenBank under accession numbers HM636440, HM636449, HM636443, HM636452 and HM636446 and CABFVS010000001 to CABFVS010000091 respectively.

Deposit in culture collection

Strain OS09^T was deposited in two different strain collections under accession numbers CSUR B624^T and DSM 23169^T.

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Conflict of Interest

None declared.

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