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TAXONOMIC DESCRIPTION
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Bartonella kosoyi sp. nov. and *Bartonella krasnovii* sp. nov., two novel species closely related to the zoonotic *Bartonella elizabethae*, isolated from black rats and wild desert rodent-fleas

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Abstract

The genus *Bartonella* (Family: *Bartonellaceae*; Order: *Rhizobiales;* Class: Alphaproteobacteria) comprises facultative intracellular Gram-negative, haemotropic, slow-growing, vector-borne bacteria. Wild rodents and their fleas harbor a great diversity of species and strains of the genus *Bartonella*, including several zoonotic ones. This genetic diversity coupled with a fastidious nature of the organism results in a taxonomic challenge that has led to a massive collection of uncharacterized strains. Here, we report the genomic and phenotypic characterization of two strains, members of the genus *Bartonella* (namely Tel Aviv and OE 1–1), isolated from *Rattus rattus* rats and *Synosternus cleopatrae* fleas, respectively. Scanning electron microscopy revealed rod-shaped bacteria with polar pili, lengths ranging from 1.0 to 2.0 µm and widths ranging from 0.3 to 0.6 µm. OE 1–1 and Tel Aviv strains contained one single chromosome of 2.16 and 2.23 Mbp and one plasmid of 29.0 and 41.5 Kbp, with average DNA G+C contents of 38.16 and 38.47 mol%, respectively. These strains presented an average nucleotide identity (ANI) of 89.9%. *Bartonella elizabethae* was found to be the closest phylogenetic relative of both strains (ANI=90.9–93.6%). The major fatty acids identified in both strains were $C_{18:1}\omega 7c$, $C_{18:0}$ and $C_{16:0}$. They differ from *B. elizabethae* in their $C_{17:0}$ and $C_{15:0}$ compositions. Both strains are strictly capnophilic and their biochemical profiles resembled those of species of the genus *Bartonella* with validly published names, whereas differences in arylamidase activities partially assisted in their speciation. Genomic and phenotypic differences demonstrate that OE 1–1 and Tel Aviv strains represent novel individual species, closely related to *B. elizabethae*, for which we propose the names *Bartonella kosoyi* sp. nov. and *Bartonella krasnovii* sp. nov.

The genus *Bartonella* belongs to the family *Bartonellaceae* of the sub-class Alpha-2-Proteobacteria. It comprises facultative intracellular Gram-negative, haemotropic, slow-growing, vector borne bacteria. Before the 1990s, this genus contained only one species, namely *Bartonella bacilliformis* [1]. Later, the reclassification of species of the genera *Grahamella* and *Rochalimaea* as members of the genus *Bartonella* [2, 3] increased the number of species belonging to this genus. Since then, the number of research studies and clinical case reports on members of the genus *Bartonella* have both increased exponentially, resulting in a large number of species from different natural sources [4]. Interestingly, several studies have emphasized the role of rodents as one of the most important natural reservoirs of species of the genus *Bartonella* [5–9]. In rodent populations, fleas have been acknowledged as the main vectors [7, 10], and it has been suggested that they may represent additional reservoirs of these bacteria [11]. To date, over 40 species and subspecies of the genus *Bartonella* have been proposed [4, 12–15], of which half have been associated with a rodent origin, including species with demonstrated

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Abbreviations: AAI, amino-acid identity; ANI, average nucleotide identity; BEACON, bacterial genome annotation comparison; CDS, coding sequences; FAME, fatty acid methyl ester; GC-FID, gas chromatography with flame ionization; GC-MS, gas chromatography mass spectrophotometry; MiGA, Microbial Genomes Atlas; PGAP, prokaryote genome annotation pipeline; RAST, rapid annotation using subsystem technology; RASTtk, rapid annotation using subsystem technology tool kit; SEM, scanning electron microscopy.
The genome sequences and annotations of these strains have been deposited in the NCBI GenBank database with the accession numbers CP031843.2 and CP031844.2 (chromosomes) and CP042964.1 and CP042965.1 (plasmids). The 16S rRNA gene sequences have been deposited under the accession numbers MN627780 and MN627781. Bacterial type strains have been deposited in the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (DSM 109921 and DSM 109922) and in the Polish Collection of Microorganisms (PCM 3037 and 3038).
A supplementary table is available with the online version of this article.

zoonotic potential [4, 16]. Remarkably, novel genetic variants of members of the genus *Bartonella* are continuously being reported from these hosts [17–20]. However, proper genomic and phenotypic characterizations of numerous detected strains are lacking, leading to an extended list of strains with unclear taxonomic status [13, 21].

Here, we formally characterized the Tel Aviv and OE 1–1 strains isolated from rodents and fleas collected from Israel. Both strains were previously associated with *Bartonella elizabethae*, as their closest genetic relative [17, 22], yet here we demonstrate that they show genomic and phenotypic differences supporting their classification as representing individual novel species. We propose the species names *Bartonella kosoyi* sp. nov. and *Bartonella krasnovii* sp. nov. for these strains, respectively.

The Tel Aviv strain was isolated from the blood of black rats (*Rattus rattus*) captured in the north-central area of Tel Aviv, Israel [17]. The approximate geographic coordinates are 32.093285° N 34.786379° E. Blood was drawn in EDTA tubes and frozen at −80 °C. Frozen blood samples were thawed and 200 µl were seeded onto chocolate agar plates and incubated aerobically at 35 °C in a humid atmosphere with 5% CO_2 [17]. The sample collection was approved by the Hebrew University's Agricultural Faculty Institutional Animal Care and Use Committee (IACUC; number AG-01–03).

Preliminary genetic characterization of the Tel Aviv strain resulted in an unclear taxonomic positioning with high genetic relatedness to both *B. elizabethae* and *B. tribocorum* [17]. In addition to the isolation of the Tel Aviv strain from *R. rattus* rats, this strain has been later molecularly detected from *Xenopsylla cheopis* fleas [23], and related-genotypes have been detected in *R. rattus* collected from other countries, such as Kenya [24], Nepal [25] and Portugal [26], and in *Bandicota* rats from Nepal [25] and Bangladesh [27]. Remarkably, the zoonotic potential of this strain has been demonstrated by its molecular detection from a woman with lymphadenopathy and fever in Tbilisi, Georgia [28].

Strain OE 1–1 was isolated from a *Synosternus cleopatrae* flea collected from a *Gerbillus andersoni* rodent, which was captured in the western area of the Negev desert, Israel. The approximate geographic coordinates were 30.750112° N 34.600138° E. The sampling of these animals was approved by the University Committee for the Ethical Care and Use of Animals in Experiments from the Ben-Gurion University of the Negev (number IL-14-03-2011). The flea was frozen at −20 °C for 5 min, superficially decontaminated with 1 ml 70% ethanol in a sterile 1.5 ml microcentrifuge tube for 5 min, and then washed three times with 1 ml of sterile Dulbecco's phosphate buffer solution 1× (PBS; Biological Industries). After the last wash, the PBS was removed and 100 µl of culture medium was added (Schneider's insect medium, Sigma-Aldrich). The flea was macerated using a sterile pestle, until a homogeneous solution was obtained. Finally, 50 µl of the macerated solution was seeded onto a chocolate agar plate and incubated at 37 °C in a humid atmosphere with 5% CO_2.

A close genetic relative of the OE 1–1 strain was firstly described from a *Gerbillus pyramidum* trapped in Egypt and imported to Japan [18]. This strain was found to be closely related to *B. elizabethae* on the basis of the results of phylogenetic analyses. In Israel, closely related organisms have been isolated and molecularly detected from several rodent species, including *Gerbillus nanus*, *G. andersoni*, *G. pyramidum, Meriones tristrami* and *Meriones crassus* [29, 30], as well as from several fleas collected from these rodents [31], reflecting a broad widespread distribution of this species in this geographical location. Recently, using multi-locus sequence analysis (MLSA) on 448 isolates of members of the genus *Bartonella* from *G. pyramidum* and *G. andersoni* and their *S. cleopatrae* associated fleas, 18 different sequence types of the OE 1–1 strain were identified and were proposed to represent a single species [22]. Additionally, this strain has been used as a model for several *in vivo* and *in vitro* ecological and evolutionary studies [9, 30, 32, 33].

For the phenotypic characterization, Tel Aviv and OE 1–1 strains were seeded on chocolate agar plates (Novamed) and incubated at 37 °C in duplicates, under the following conditions: (i) aerobic (i.e. standard incubator); (ii) capnophilic (i.e. 5% CO_2) in a CO_2 incubator (Thermo Forma II Water Jacket CO_2 Incubator, Thermo Scientific); (iii) microaerophilic (i.e. 6.2–13.2% O_2, 2.5–9.5% CO_2) using CampyGen bags (Oxoid, Thermo Scientific); and (iv) anaerobic (i.e. <0.1% O_2, 7.0–15.0% CO_2) using AnaeroGen bags (Oxoid). Both strains grew optimally under increased CO_2 conditions, capnophilic and microaerophilic, exhibiting round and smooth colonies (≥1 mm) after 4–5 days (Table 1). The strains were demonstrated to be strictly capnophilic and microaerophilic, since no growth was obtained under aerobic or anaerobic conditions. Notably, Clarridge and colleagues [34] have shown that other species of the genus *Bartonella*, including *Bartonella elizabethae*, *Bartonella henselae* (Houston-3 strain), *Bartonella clarridgeiae*, *Bartonella quintana* and *Bartonella vinsonii* have the capacity to grow under strictly aerobic conditions (with no increase of CO_2). Moreover, Kesnerova and collaborators [12], also recorded slow growth of *Bartonella apis* under aerobic conditions, but failed to obtain colonies of *B. henselae* (Houston-1 strain) under these conditions.

The bacteria were subjected to light microscopy and scanning electron microscopy (SEM) to evaluate the shape, cell length and width. For light microscopy, the bacteria were stained with Gram stain set (Hy-labs) and visualized using 100× objectives. For SEM, cultures from chocolate agar plates were collected and fixed in a solution containing 4% formaldehyde, 2.5% glutaraldehyde and 0.1 M cacodylate buffer for 1 h, and then washed five times in phosphate buffer. Thereafter, 1% osmium tetroxide was added and the solution was incubated for 1.5 h. The bacteria were then dehydrated through a graded ethanol series (25–100%), and dried with a critical point dryer (Quorum K850, Quorum Technologies) with CO_2 and coated with iridium for 20 s (Quorum Spatter coater Q150 T ES, Quorum Technologies). Finally, the bacterial cells were visualized by SEM (JSM-7800F, JEOL). SEM images were analyzed using the ImageJ 1.48 v software (NIH)

Table 1. Phenotypic and genomic descriptions of two novel species of the genus *Bartonella*

***Bartonella* strain**	**Tel Aviv**	**OE 1-1**
Gram staining	Negative	Negative
Morphology	Rods	Rods
Length (average, μm±SD)	1.6±0.30	1.4±0.22
Width (average, μm±SD)	0.4±0.06	0.4±0.04
Incubation time at 37 °C with increased CO_2 (2.5–9.5%; for a 1–2 mm colony)	5 days	4 days
Chromosome (large circular sequence)	1	1
Estimated chromosomal length (bp)	2 234 681	2 157 564
DNA GC content (mol%)	38.47	38.16
Plasmids (small circular sequence)	1	1
Plasmid estimated length (bp)	41483	29057
Protein coding genes (CDS)	1777	1675
Pseudogenes	146	149
tRNA genes	42	42
rRNA genes	6	6
ncRNAs	4	4
rRNA operons	2	2
Predicted prophage-related genes	193	115
Major transporter families*	ABC transporters, Tol–Pal system, MFS transporters, RND, HlyC/CorC, MATE AAA ATPase	ABC transporters, Tol–Pal system, MFS transporters, RND, HlyC/CorC, MATE, AAA ATPase
Type III secretion systems*	YopJ-like	YopJ-like
Type IV secretion systems*	Three chromosomal (i.e. VirB-like, Trw, Vbh), one plasmidic (i.e. conjugal system)	Three chromosomal (i.e. VirB-like, Trw, Vbh), one plasmidic (i.e conjugal system)
Adhesin genes (i.e. *badA*)	One cluster (six copies)	One cluster (four copies)
Filamentous hemagglutinin-related genes	10	19
Haemin binding-related genes	6	5
Bacterial DNA polymerases	I and III	I and III
RNA polymerase sigma factors	RpoD, RpoE, RpoH1, RpoH2, and sigma-54	RpoD, RpoE, RpoH1, RpoH2, and sigma-54

*One or more genes related to each mentioned protein family or system were identified in the genomes.

[35] and the estimates of the bacterial length and width were calculated from these images. Under light microscopy, both strains showed Gram-negative staining. Under SEM, cells of both strains appeared as small rods (Table 1, Figs 1 and 2). Both strains exhibited polar pili without flagella, forming tight aggregates of the bacteria (Figs 1 and 2). Interestingly, similar structures have been identified earlier in the rodent-associated species, *B. tribocorum* [36], *Bartonella alsatica* [37], *Bartonella fuyuanensis* [38], *Bartonella jaculi, Bartonella callosciuri, Bartonella pachyuromydis* and *Bartonella acomydis* [39]; but not observed or reported in many other species, including the rodent-associated species *Bartonella japonica*, *Bartonella silvatica* [40], and *Bartonella heixiaziensis* [38].

For biochemical and metabolic characterization, the strains were tested with the BBL Crystal Enteric/Nonfermenter (BD Becton Dickinson), API 20E and Rapid ID 32A (BioMerieux) identification systems, following the manufacturers' instructions. Catalase enzymatic reaction was performed using hydrogen peroxide (3%). Both strains were negative for oxidase, catalase, urease, indole production, nitrate reduction and acetoin production (Voges–Proskauer reaction). Both strains were inert to the majority of the carbohydrates tested, yet both exhibited a variable reaction (i.e. positivity was observed only in one of the kits used) for arabinose, while the OE 1–1 strain showed variable reactions to sucrose and citrate, and the Tel Aviv strain to sorbitol, melibiose, rhamnose and

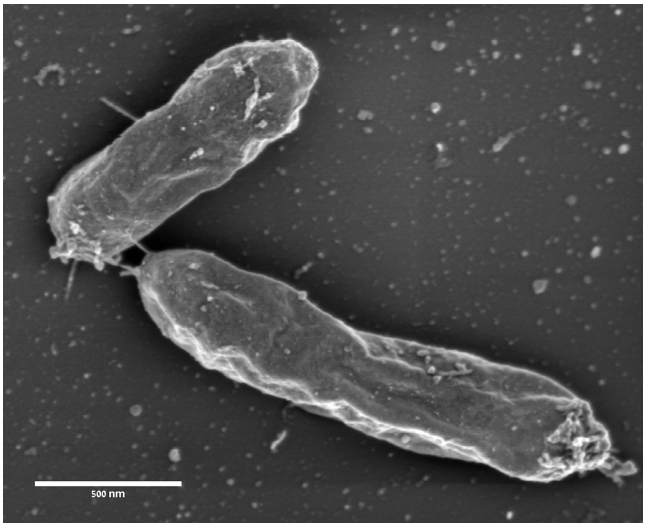

Fig. 1. Scanning electron microscopy of the OE 1–1 strain (*Bartonella krasnovii* sp. nov.). A 500 nm scale bar is shown within the image.

glucose. Both strains were negative for the hydrolysis of the *p*-nitrophenyl substrates, except for *p*-nitrophenyl phosphate (both positive). Both strains were positive for alkaline phosphatase. Amino acid arylamidase activities were recorded for arginine, phenylalanine, leucine, tyrosine, alanine and glycine in both strains; while they differed with respect to the arylamidase activity to histidine and serine (only positive for the OE 1–1 strain). Both strains were negative for arylamidase activity to proline, leucyl glycine, pyroglutamic acid and glutamyl-glutamic acid. The recorded biochemical profiles resemble the typical profiles of other species of the genus *Bartonella* with validly published names. Table S1 (available in the online version of this article) summarizes 12 key tests that can assist in the discrimination of the OE 1–1

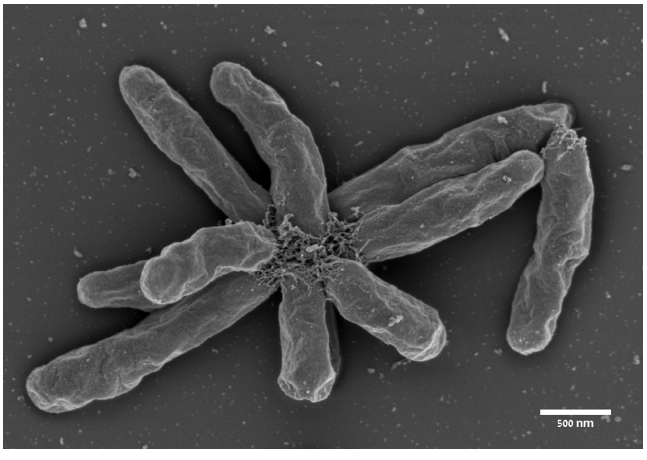

Fig. 2. Scanning electron microscopy of the Tel Aviv strain (*Bartonella kosoyi* sp. nov.) showing rods interconnected with polar pili. A 500 nm scale bar is shown within the image.

Table 2. Percentages (±SD) of the major fatty acid methyl ester (FAME) compositions of the two novel species of the genus *Bartonella* compared with the *B. elizabethae* ATCC 49927^T control (their closest relative)

Strain/FAME	Strain Tel Aviv	Strain OE 1–1	*B. elizabethae* ATCC 49927
$C_{15:0}$	0.0	0.0	1.5 (±0.5)
$C_{16:0}$	19.8 (±3.9)	22.0 (±3.5)	21.7 (±2.9)
$C_{16:1}\omega7c$	0.9 (±0.5)	0.7 (±0.4)	1.0 (±0.4)
$C_{17:0}$	0.5 (±0.5)	0.3 (±0.3)	10.4 (±4.0)
$C_{17:1}\omega6c$	0.6 (±0.6)	0.1 (±0.1)	1.8 (±1.0)
$C_{18:0}$	24.4 (±3.8)	24.9 (±4.1)	18.4 (±4.5)
$C_{18:1}\omega7c$	50.0 (±5.0)	50.8 (±3.4)	44.0 (±3.4)
Unidentified FAMEs	4.1	1.2	1.1

and Tel Aviv strains from the majority of other species with validly published names.

The analysis of the bacterial fatty acid methyl ester (FAME) composition was carried out from chocolate agar cultures of OE 1–1 and Tel Aviv strains, as well as *B. elizabethae* ATCC 49927^T (as control). The strains were incubated at 37 °C with 5% CO_2 atmosphere for 5–6 days and the bacterial masses were collected from the plates into individual glass flasks. Then, the bacterial masses (by duplicates) were lyophilized and their FAME compositions were measured by gas chromatography with flame ionization detector (GC-FID) and mass spectrophotometry (GC-MS). The tentative FAME identification was assessed using in-house FAME standards and in comparison to the retention times of the *B. elizabethae* control, and its previous FAME composition descriptions [34, 41]. The FAME compositions were similar for both strains with a distribution of $C_{18:1}\omega7c > C_{18:0} > C_{16:0}$ (Table 2). Notably, OE 1–1 and Tel Aviv strains can be distinguished from *B. elizabethae* by differences in the amount of $C_{17:0}$ and $C_{15:0}$ (Table 2).

Genomic DNA extraction was performed from chocolate agar cultures using commercial kits (Illustra Tissue and Cells, GE Healthcare). The genomes were sequenced using MiSeq Illumina (2×250 bp paired-end) and MinION Nanopore (Oxford Nanopore Technologies; average of 7000 bp long reads) technologies. A total of 1017594–1238730 sequenced Illumina MiSeq reads and 43612–57779 Nanopore reads (median read length of 7078–9043 bp) were obtained. Hybrid *de novo* assembly was performed with UNICYCLER pipeline [42]. Each genome was assembled into a circular contig (i.e. chromosome) of 2.16 and 2.23 Mbp and a small circular contig (i.e. plasmid) of 29.0 and 41.5 kbp for the OE 1–1 and Tel Aviv strains, respectively. The genomic features of each strain are shown in Table 1.

The genomes were annotated using classical RAST [43], RASTtk [44], PROKKA [45], and PGAP [46] pipelines. The annotation outputs were compared using BEACON [47]. The PGAP was defined as the reference annotation. Hypothetical

protein and phage-related gene annotations were improved from the reference annotation using the extended annotation output from BEACON. Furthermore, the protein coding genes were compared with those of the annotated *Bartonella grahamii* as4aup [48] using BLASTp to improve the identification of well-characterized virulence factor genes (e.g. the Trw locus and putative filamentous haemagglutinin genes). Similarly, the genomes of members of the genus *Bartonella* were compared to the Alphaproteobacteria reference genome of *Brucella abortus* strain 2308 [49], using BLASTp. Hypothetical genes in the genomes of members of the genus *Bartonella* that matched well-characterized functional genes in the reference genome from *Brucella abortus* strain 2308 (i.e. matches with ≥50% identity, e-value ≤10^{-6}) were defined as 'putative' genes in the *Bartonella* final annotations. The final gene features are summarized in Table 1.

As mentioned above, the hybrid *de novo* assembly protocol resulted in an additional small circular contig per strain. Thus, the presence of plasmids was confirmed by the S1-nuclease protocol for detecting and sizing large plasmids, as described previously [50]. Accordingly, agarose plugs were prepared from a fresh solution of both strains and digested with S1-nuclease (Thermo Scientific) at 37 °C for 45 min. The plugs were run in CHEF Mapper XA (Bio-Rad Laboratories) with the following two-stage protocol: stage 1, switch times 2–10 s with a run time of 19 h; stage 2, switch times 20–25 s with a run time of 6 h. Both stages were run at 6.0 V cm^{-1}, with angles of 60 and −60 degrees each step, in 0.5% Tris-borate EDTA buffer at 14 °C. Gels were stained with Sybr Gold (Invitrogen) for 24 h. Gels were inspected using ImageLab software (Bio-Rad). *Salmonella* ser. Braenderup H9812 plugs digested with *XbaI* (New England BioLabs) were used as a molecular marker in each run. The S1-nuclease protocol confirmed that OE 1–1 and Tel Aviv strains harbor a circular plasmid with a linearized length of approximately 29 and 40 kb, respectively (Fig. 3), in full accordance with the obtained lengths of the sequences of the small contigs (Table 1).

The small contigs obtained from each strain were confirmed to be plasmid sequences through the gene annotation pipelines. Accordingly, these sequences harbor plasmid-related genes, such as the plasmid replication protein gene (*repC*), plasmid partitioning protein gene (*parA*) and conjugal protein genes (*trb*, *tra*). Other genes encoded in both plasmids are resolvase, recombinase, MATE family efflux transporter, AAA family ATPase, helix–turn–helix domain-containing proteins and several hypothetical proteins in both plasmids. The Tel Aviv strain plasmid also encodes a FAD-binding protein, DeoR family transcriptional regulator, phenylacetate-CoA ligase and ATP-grasp domain-containing proteins. The plasmids of the OE 1–1 and Tel Aviv strains were named pOE11-1 and p-TLV-1, respectively.

Plasmids have also been described in *Bartonella grahamii* [51], *B. tribocorum* [52], *Bartonella schoenbuchensis* [53], and *Bartonella rattaustraliani* [54]. These reference plasmids only covered 1.6–11.9% of the novel strains' associated plasmids (Table 3). The average nucleotide identity on the covered

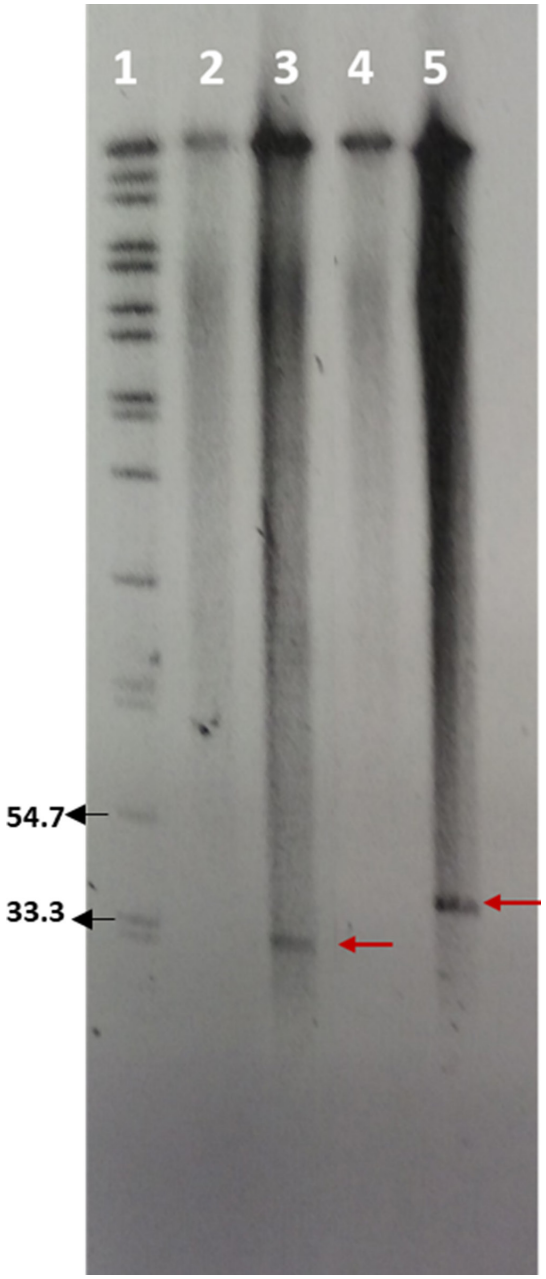

Fig. 3. Determination and sizing of plasmids from the two novel strains of members of the genus *Bartonella*. Bacteria were prepared in agarose plugs, lysed with proteinase K, treated with S1-nuclease and ran in a PFGE. Controls were prepared in parallel without treatment with S1-nuclease. S1-nuclease treatment converted large circular supercoiled plasmids to linear molecules that migrated as discrete bands. Untreated bacterial plasmids (circular and supercoiled) migrated much more slowly than linear forms, being indistinguishable from the migration of the bacterial chromosomes (upper bands near the inoculation well). Line 1: *Salmonella* ser. Braenderup H9812 plugs digested with *XbaI* (as molecular marker); 2: OE 1–1 strain untreated; 3: OE 1–1 strain treated with S1-nuclease; 4: Tel Aviv strain untreated; 5: Tel Aviv strain treated with S1-nuclease. Red arrows highlight the linearized plasmids.

Table 3. *Bartonella* plasmids comparison. Average nucleotide identity (OrthoANIu) and coverage of the Tel Aviv (pTLV-1) and OE 1–1 (pOE11-1) strains-associated plasmids and the reference plasmids from members of the genus *Bartonella*

		pTLV-1 (41,483 bp)		pOE11-1 (29,057 bp)	
Reference plasmid (GenBank accession numbers)	**Length (bp)**	**OrthoANIu (%)**	**Coverage (%)**	**OrthoANIu (%)**	**Coverage (%)**
Bartonella tribocorum pBT (AM260524.1)	23343	79.75	3.65	80.54	10.4
Bartonella grahamii pBGR3 (CP001563.1)	28192	76.36	4.83	75.35	9.14
Bartonella schoenbuchensis pML (CM001845.1)	57959	76.38	7.54	68.6	11.88
Bartonella rattaustraliani pNH4 (FJ605483.1)	11227	59.7	1.6	85.70	2.06
pOE11-1/pTLV-1	29057/41483	93.77	44.81	93.77	64.01

sequence ranged from 59.7–85.7% (Table 3). The pTLV-1 and pOE11-1 plasmids were found to be closely related, sharing 44.8–64.0% with a >93% sequence identity.

Analysis of the partial 16S rRNA gene sequences was performed with all the sequences of type strains of species of the genus *Bartonella* according to the List of Prokaryotic names with Standing in Nomenclature (LPSN; last accessed on October 17, 2019: http://www.bacterio.net/bartonella.html). *Bartonella peromysci* and *Bartonella talpae* were not included due to lack of 16S rRNA type sequences of these strains. The analysis included a 1270 bp fragment of 16S rRNA gene sequences (85% of the complete gene). A Bayesian inference tree was reconstructed using BEAST v1.8.4 [55]. Results from previous phylogenetic studies have indicated that the 16S rRNA gene presents a low discriminatory power for this genus [56]. Nevertheless, the obtained tree topology showed that both OE 1–1 and Tel Aviv strains are more closely related to the *Bartonella* species, *B. elizabethae*, *B. tribocorum, Bartonella pachyuromydis* and *Bartonella florencae* with posterior probability >0.8 (Fig. 4a). In addition, a Bayesian inference tree was reconstructed using a 316 bp fragment of the most common gene target for phylogenetic inference on *Bartonella*, the citrate synthase gene (*gltA*). The resulting tree topology confirmed the positioning of these novel strains in close phylogenetic proximity to *B. elizabethae* and *B. tribocorum* (Fig. 4b).

Whole-genome average nucleotide identity (ANI) was calculated between genomes of members of the genus *Bartonella* using OrthoANIu [57] available online (last accessed on October 24, 2019: https://www.ezbiocloud.net/tools/ani). A whole-genome-based phylogenetic tree was reconstructed using RAxML [58] implemented in PATRIC [59]. The analysis included all the species of the genus *Bartonella* with validly published names with complete genomes, and held 913 shared protein genes for a total of 301917 nucleotide positions from a total of 49

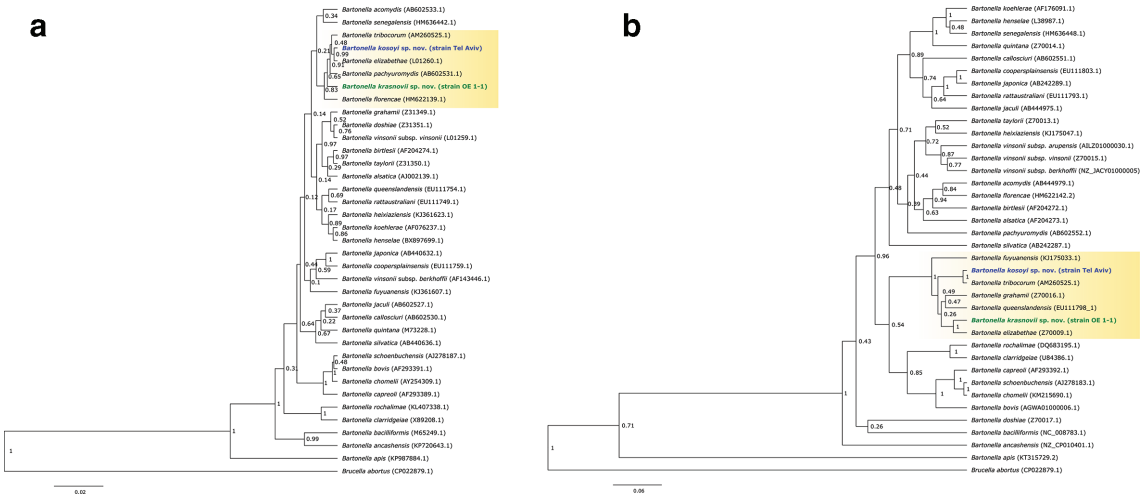

Fig. 4. Single-gene-based phylogeny. (a) Phylogenetic tree of species of the genus *Bartonella* based on partial fragment of the 16S rRNA gene (1,270 bp). (b) Phylogenetic tree of species of the genus *Bartonella* based on partial fragment of the citrate synthase gene (*gltA*; 316 bp). Bayesian Inference trees were reconstructed using BEAST. Posterior probabilities are shown at the branches. Bar, substitutions per nucleotide position. Strain Tel Aviv (*Bartonella kosoyi* sp. nov.) is indicated in blue type and strain OE 1–1 (*Bartonella krasnovii* sp. nov.) in green type. The most closely related species of the genus *Bartonella* to the novel strains are highlighted within a colored box. *Brucella abortus* strain MC was used as an outgroup. The GenBank accession numbers of each reference species are indicated in parentheses.

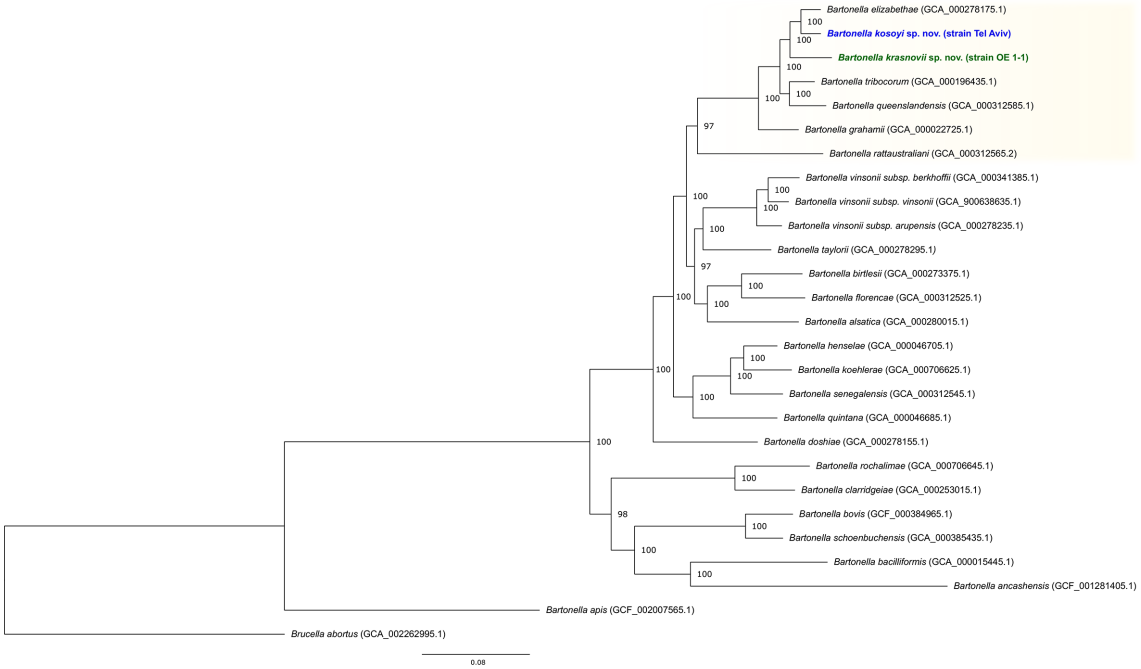

Fig. 5. Whole-genome-based phylogeny. Phylogenetic tree of species of the genus *Bartonella* based on 931 shared protein genes and 301 917 nucleotide positions. The tree was reconstructed using the RAxML method. Strain Tel Aviv (*Bartonella kosoyi* sp. nov.) is indicated in blue type and strain OE 1–1 (*Bartonella krasnovii* sp. nov.) in green type. Percentages of bootstrap values are shown at the branches. Bar, substitutions per nucleotide position. *Brucella abortus* strain MC was used as an outgroup. The assembly accession numbers of each reference species are indicated in parentheses.

genomes. *Brucella abortus* strain MC (GenBank accession number: CP022879-80) was used as the outgroup. The phylogenetic tree analysis revealed that the two novel strains clustered together with *Bartonella elizabethae* (Fig. 5). The obtained OrthoANIu values between the two novel strains was 89.9%, with a coverage genome of 55.9–58.18%. The OrthoANIu values obtained between the novel strains and the most closely related reference species are shown in Table 4. Other more distantly related members of the genus *Bartonella* showed ANI values below 83%, with *B. apis* being the most distant *Bartonella* relative described to date (OrthoANIu~69%). Accordingly, the tree topology and OrthoANIu values demonstrated that *Bartonella elizabethae* is the closest phylogenetically validated reference species for both strains, followed by *B. tribocorum*. Remarkably, these reference genomes covered only a 53.4–65.0% of the novel genomes. Several studies have supported the use of 95% of ANI

Table 4. Average nucleotide identity (ANI) and coverage of the novel strains and their closest relatives

	Tel Aviv strain		OE 1–1 strain	
Reference genome (genome accession number)	**OrthoANIu (%)**	**Coverage (%)***	**OrthoANIu (%)**	**Coverage (%)***
Bartonella elizabethae (NZ LR134527)	93.61	59.96	90.92	53.37
Bartonella tribocorum (NC 010161)	92.46	65.03	88.74	58.17
Bartonella queenslandensis (GCA_000312585.1)	90.18	48.08	88.56	48.52
Bartonella grahamii (NC 012846)	87.84	54.88	87.08	55.47
Bartonella florencae (GCA_000312525.1)	83.5	47.75	83.77	47.69
Bartonella vinsonii subsp. *berkhoffii* (NC 020301)	82.81	44.12	82.70	44.96
Bartonella birtlesii (NZ CM001557)	82.9	42.94	83.00	43.97
Bartonella rattaustraliani (GCA_000312565.2)	81.68	42.73	81.49	43.63

*Percentage of genome of the novel strain included in the OrthoANIu analysis.

as a threshold for the differentiation of bacterial species [60–62]. Therefore, genomic evidence supports the description of both OE 1–1 and Tel Aviv strains as representing novel species.

DESCRIPTION OF *BARTONELLA KOSOYI* SP. NOV.

Bartonella kosoyi (ko.so′yi. N.L. masc. gen. n. *kosoyi* of Kosoy, in honor of Michael Kosoy from the Centre for Disease Control, USA, for his extensive contribution to the study of rodent-associated species of the genus *Bartonella*).

Bacterial cells are rods, 1.1–2.0 µm long and 0.3–0.6 µm wide, and show polar pili. They present optimal growth under capnophilic conditions (5% CO_2) at 37 °C. Colonies grown on chocolate agar are smooth and circular. Growth to achieve 1.0–2.0 mm colonies occurs in 5 days at 37 °C. Negative for oxidase, catalase, urease, indole production, acetoin production and nitrate reduction. Hydrolyzes *p*-nitrophenyl phosphate, but no other *p*-nitrophenyl substrates, including *p*-nitrophenyl bis phosphate. Exhibits arylamidase activities to arginine, phenylalanine, leucine, tyrosine, alanine and glycine, but not to proline, leucyl glycine, pyroglutamic acid, histidine, glutamyl-glutamic acid or serine. The major fatty acids are $C_{18:1}\omega7c$, $C_{18:0}$ and $C_{16:0}$.

The type strain carried a single circular chromosome of 2.23 Mb and a circular plasmid of 41.5 kb, with an average DNA G+C content of 38.47 mol%. The most closely related species of the genus *Bartonella* with a validly published name is *B. elizabethae*, which can be distinguished from this strain by the $C_{15:0}$ and $C_{17:0}$ FAME compositions (present largely in *B. elizabethae*), genetic composition (pairwise ANI of 93.6%), and by the *p*-nitrophenyl bis phosphate and serine arylamidase reactions (both positive only in *B. elizabethae*). *Bartonella kosoyi* type strain isolates have been deposited in the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (DSM 109922) and in the Polish Collection of Microorganisms (PCM 3038).

DESCRIPTION OF *BARTONELLA KRASNOVII* SP. NOV.

Bartonella krasnovii (kras.no′vi.i. N.L. masc. gen. n. *krasnovii* of Krasnov, in honor of Boris Krasnov from the Ben-Gurion University of the Negev, Israel for his extensive contribution to the study of the ecology and evolution of desert rodents and associated fleas).

Bacterial cells are rods, 1.0–1.9 µm long and 0.3–0.4 µm wide, and show polar pili. Optimal growth is obtained under capnophilic conditions (5% CO_2) at 37 °C. Colonies grown on chocolate agar are usually smooth and circular (for the type strain) but other strains may present different morphotypes (e.g. creamy, dry, flat, raised, etc.). Growth to achieve 1.0–2.0 mm colonies occurs in 3–4 days at 37 °C. Negative for oxidase, catalase, urease, indole production, acetoin production and nitrate reduction. Hydrolyzes *p*-nitrophenyl phosphate, but no other *p*-nitrophenyl substrates, including *p*-nitrophenyl bis phosphate. Exhibits arylamidase activities to arginine, phenylalanine, leucine, tyrosine, alanine, glycine, histidine and serine, but not to proline, leucyl glycine, pyroglutamic acid or glutamyl glutamic acid. The major fatty acids are $C_{18:1}\omega7c$, $C_{18:0}$ and $C_{16:0}$.

The type strain carried a single circular chromosome of 2.16 Mb and a circular plasmid of 29.0 kb, with an average DNA G+C content of 38.16 mol%. The most closely related species of the genus *Bartonella* with a validly published name is *B. elizabethae*, which can be distinguished from this strain by the $C_{15:0}$ and $C_{17:0}$ FAME compositions (largely present in *B. elizabethae*), genetic composition (pairwise ANI of 90.92%), and by the *p*-nitrophenyl bis phosphate reaction (positive only in *B. elizabethae*). *Bartonella krasnovii* type strain isolates have been deposited in the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (DSM 109921) and in the Polish Collection of Microorganisms (PCM 3037).

DATA DEPOSITION

The genomes of these strains have been deposited in the NCBI GenBank database with the accession numbers CP031843.2 and CP031844.2 (chromosomes) and CP042964.1 and CP042965.1 (plasmids).

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Conflicts of interest
The authors declare no conflicts of interest.

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