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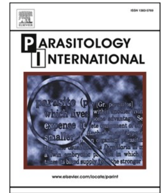

Eutrichophilus cordiceps Mjöberg, 1910 (Ischnocera: Trichodectidae) in Spiny Tree Porcupines (*Coendou villosus*): New locality records and the first molecular evidence of association with *Bartonella* sp.

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ABSTRACT

The chewing louse genus *Eutrichophilus* Mjöberg has 19 species only associated with porcupines (Rodentia: Erethizontidae). Of these species, *E. cercolabes*, *E. cordiceps*, *E. emersoni*, *E. minor*, *E. moojeni*, and *E. paraguayensis* have been recorded in Brazil. In the present study, we report *E. cordiceps* for the first time in the São Paulo State (Bauru Municipality) and for the second time in the Santa Catarina State (Lages Municipality), providing scanning electron images and light microscopy for the eggs, as well as the first molecular data (18S rRNA) for the genus. Additionally, *Bartonella* sp. was detected for the first time in this chewing lice species.

1. Introduction

The bacterial genus *Bartonella* encompasses facultative Gram-negative that infect primarily erythrocytes and endothelial cells. Rodents are considered the main reservoirs of this bacteria, harboring high diversity of these vector-borne agents [7], as fleas, mosquitoes, flies, ticks, and lice, depending on the species involved.

Lice species (Anoplura, Amblycera and Ischnocera) constitute a very diverse group of insects, exclusively adapted to parasitism [37]. Due this parasitism, some species have been associated with pathogenic microorganisms, for example the human body lice, *Pediculus humanus* L., 1758 (Anoplura: Pediculidae), that is vector for *Bartonella quintana*, *Rickettsia prowazekii* and *Borrelia recurrentis*, the etiological agents of the trench fever, epidemic typhus, and the louse-borne relapsing fever, respectively [5,6,14]. On the other hand, some chewing lice species can serve as intermediate hosts for the cestode species *Dipylidium caninum*, as well as can transmit some filariasis [41].

The lice genus *Eutrichophilus* Mjöberg, 1910 is a chewing louse genus

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that is only associated with porcupines (Rodentia: Erethizontidae) [30,45]. Timm and Price [45,46] reviewed this genus, listing 19 valid species. Of these, eight species have been recorded parasitizing different porcupine species in Brazil ([36,45]; [50]): *Eutrichophilus cercolabes* Mjöberg, 1910, *Eutrichophilus cordiceps Mjöberg, 1910*, *Eutrichophilus emersoni* Timm & Price, 1994, *Eutrichophilus lobatus* Ewing, 1936, *Eutrichophilus mexicanus* (Rudow, 1866), *Eutrichophilus minor* Mjöberg, 1910, *Eutrichophilus moojeni* Werneck, 1945, and *Eutrichophilus paraguayensis* Tim & Price, 1994.

In the present study, we provided new locality records for the species *E. cordiceps*, scanning electron images, additional light microscopy for the eggs, and the first molecular data (18S rRNA) for the genus. Additionally, we performed molecular assays for *Bartonella* sp. screening and characterization in these ectoparasites, aiming at better understanding this pathogen-association relationship.

2. Materials and methods

2.1. Material collection and identification

The specimens studied in the present study came from two different localities. (1) During the daily routine at the Municipal Zoo of Bauru Municipality, São Paulo State, zookeepers observed high infestation of lice in a couple of Spiny Tree Porcupine, *Coendou villosus* F. Cuvier, 1823 (Rodentia: Erethizontidae). The zookeepers were instructed to collect and store these ectoparasites (62 specimens) in 70% ethyl alcohol for identification. The two animals were treated with Doramectin (single dose of 0.5 mg/kg administered by subcutaneous injection) and Selamectin (single dose of 15 mg/kg for topical administration, administered one week after Doramectin application). The Spiny Tree Porcupine couple did not present injuries or any other clinical sign that could be a consequence of parasitism of these ectoparasites. (2) Lice (94 specimens) were collected and stored in 70% ethyl alcohol during a necropsy of three Spiny Tree Porcupines road-killed and presenting high lice infestation in the Lages Municipality, Santa Catarina State.

After the two samplings, the lice specimens were sent to the Laboratório de Coleções Zoológicas (LCZ), Instituto Butantan, São Paulo, SP, Brazil. Furthermore, for a complete understanding of the specimens, we analyzed the material using three methods: (1) Light microscopy, (2) Scanning Electron Microscopy (SEM), and (3) molecular analysis for pathogens screening, focusing on *Bartonella* detection.

Part of the material was slide-mounted (five males, five females, and 2 nymphs of each sampling) and analyzed up to genus level, based on Price et al. [37], as well as up to species level, based on keys and images made by Timm and Price [45,46]. The light microscopy images were taken using a Leica DFC 500 digital camera coupled to a Leica DM4000B optical microscope. Extended focal range images were composed using Leica Application Suite version 2.5.0. Another part of the material (five males, five females, and 2 nymphs of each sampling) was prepared for SEM, using the Digital Scanning Microscope FEI, Quanta 250, at the Laboratório de Biologia Celular, Instituto Butantan, São Paulo, SP, Brazil. The figures were prepared using Adobe Photoshop v. 13.0 software.

All the materials were deposited in the Entomological Collection of the Butantan Institute (IBSP), São Paulo municipality, São Paulo State, Brazil, under the accession numbers IBSP-Ent 14,636–14,640.

2.2. Molecular analysis

The remaining of the specimens (30 males, 26 females, and 14 nymphs from Lages Municipality, and 11 males, 23 females, and four nymphs from Bauru Municipality) were subjected to DNA extraction, using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following manufacturer instructions. The chewing lice were individually placed in 1.5 mL microtubes and punctured in the distal region of the abdomen with a sterile needle. After the DNA extraction, lice were recovered, slide-mounted, and kept as vouchers in the IBSP-Ent Collection.

A conventional PCR (cPCR) to amplify a fragment of ~800-pb of 18S rRNA gene with primers Mite18S-1F (3′-ATATTGGAGGGCAAGTCTGG-5′) and Mite18S-1R (3′-TGGCATCGTTTATGGTTAG-5′) [34] was implemented as internal control of DNA extraction. The samples were also submitted to a cPCR targeting ~500-pb fragment of the mitochondrial cytochrome *c* oxidase subunit 1 (COI) using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) [16]. Negative (Milli-Q water free of DNA) and positive (pool of dust mites) controls were used for each reaction. In order to assure that there is no remaining host DNA in the samples, a cPCR assay targeting a ~ 400-pb fragment for the *gapdh* gene was performed using the protocol described in Birkenheuer et al. [4].

An initial screening for *Bartonella* spp. was performed using the qPCR described by André et al. [2], which targets an 83 bp fragment of the NADH dehydrogenase gamma subunit gene (*nuoG*). All samples were evaluated in duplicate, and when difference greater than 0.5 in the Cq values was observed between both replicates, we re-tested the sample in triplicates. In order to construct a standard curve, serial dilutions of 1.0×10^7 to 1.0×10^0 copies of a plasmid (pIDTSMART; Integrated DNA Technologies, Coralville, IA, USA) encoding a fragment of the *Bartonella henselae* gene cited above was implemented. The number of plasmid copies was determined with the formula (XG/μL DNA/ [Plasmid Length (BP) × 660]) × 6.22×10^{23} × plasmid copies/μL, and the amplification efficiency (E) was calculated according to the slope of the standard curve using the formula $E = 10^{-1/slope}$ [8]. The qPCR assays were conducted in a C1000-CFX96 thermal cycler (BIORAD®) in the Vector-Borne Bioagents Laboratory (VBBL), São Paulo State University (UNESP), Jaboticabal, SP, Brazil.

Positive samples by qPCR were then subjected to cPCR based on six different *Bartonella* molecular markers: *nuoG* [10], *rpoB* [40], *Pap31* [27], *gltA* [32], *groEL* [35,49], and *ftsZ* [35]. Negative (Milli-Q water free of DNA) and positive (*Bartonella henselae*) (Dias et al *[12]*) controls were used for each reaction.

All PCR products with concentrations above 20 ng/μL were selected and purified with ExoSap-IT (GE Healthcare Pittsburgh, PA). Sanger sequencing was performed at the "Centro de Pesquisa sobre Genoma Humano e Células Tronco do Instituto de Biociências da USP", São Paulo, SP, Brazil. Obtained sequences were assembled with Sequencing Analysis 5.3.1 and submitted to BLAST analysis [1] to infer similarities with other lice and *Bartonella* sequences available in GenBank. Different haplotypes were visually discriminated after an alignment using the CLUSTAL W algorithm [44] implemented in Geneious R11 [22].

2.3. Phylogenetic analysis

The obtained *Bartonella* sequences were aligned with other homologous sequences retrieved from the database (GenBank) using the MAFFT software (available online: https://mafft.cbrc.jp/alignment/server/index.html) [51] and edited via Bioedit v. 7.0.5.3 [19]. W-IQ-Tree software was used to select the best evolutionary model following the BIC criterion and to construct phylogenetic analyses with the Maximum Likelihood method (available online: http://iqtree.cibiv.univie.ac.at/) [47]. Clade support was evaluated through 1000 bootstrap replicates. The phylogenetic trees were edited using Treegraph 2.0.56–381 beta software [43].

2.4. Haplotype network

A genetic diversity analysis for the *nuoG Bartonella* sequences was performed with the sequences obtained in this study aligned to phylogenetically close sequences of *Bartonella* spp. The alignment was performed using MAFFT as explained above. The alignment was also used to calculate the nucleotide diversity (π), diversity of haplotypes (Dh), number of haplotypes (h), and the average number of nucleotide differences (K), using DnaSP v5 software [26]. The Haplotype Network was

constructed in Population Analysis with Reticulate Trees v1.7 (popART) software [25], using the TCS network [9].

3. Results

3.1. Morphological identification

All the collected specimens (21 males, 33 females, 8 nymphs in Bauru Municipality in the two hosts; 40 males, 36 females, 18 nymphs in Lages Municipality in the three hosts) were identified as *Eutrichophilus cordiceps* Mjöberg, 1910 (Figs. 1 and 2). Morphologically, this species can be separated from the other *Eutrichophilus* species because of the (1) the large size of the adult stages (female and male); (2) the size of the accessory tergal plate; (3) the unique shape of the genitalia in males; (4) the shape and chaetotaxy of gonapophyses, subgenital plate and sternites II and III in females [45,46]. Fig. 2 shows an egg inside the female, and it is possible to observe the hive-shaped ornamentations on it.

3.2. Molecular analysis

Out of 108 lice specimens from which DNA was extracted, 95 (87.1%) were positive for the endogenous control (18S rRNA). No samples were positive in the PCR targeting the COI gene. It is worth mentioning that the 14 DNA-negative samples were obtained from nymphs from both localities. Sequencing of *gapdh* was negative for all samples, proving there is no host DNA in any of the samples.

Of these 95 positive samples for the endogenous control, five males and five females from each sampled locality, and the four positive nymphs (three from Lages and one from Bauru) were chosen by convenience and submitted for sequencing. Twenty-four sequences were obtained and shown to be identical to each other, representing a single haplotype (836 bp). This single haplotype was deposited in the GenBank database (accession number OR589777). BLAST analysis of this

Fig. 2. Light microscopy image of the eggs inside a female of *Eutrichophilus cordiceps* Mjöberg, 1910. The black arrow highlights the ornamentation on the surface of the egg. Scale bar: 100 μm.

sequence showed similarities with other genera in the Trichodectidae. The obtained haplotype was 99.09% (327/330-bp, e-value: 6e-164) identical to *Neotrichodectes pallidus* (OP831279) and 97.96% (336/343-bp, e-value: 2e-164) identical with a sequence for an unidentified Trichodectidae (ON964865).

After confirming the positivity of the endogenous control, the 95 samples were subjected to qPCR for *Bartonella* sp. based on the *nuoG* gene. Ten samples were positive, whose quantification ranged from

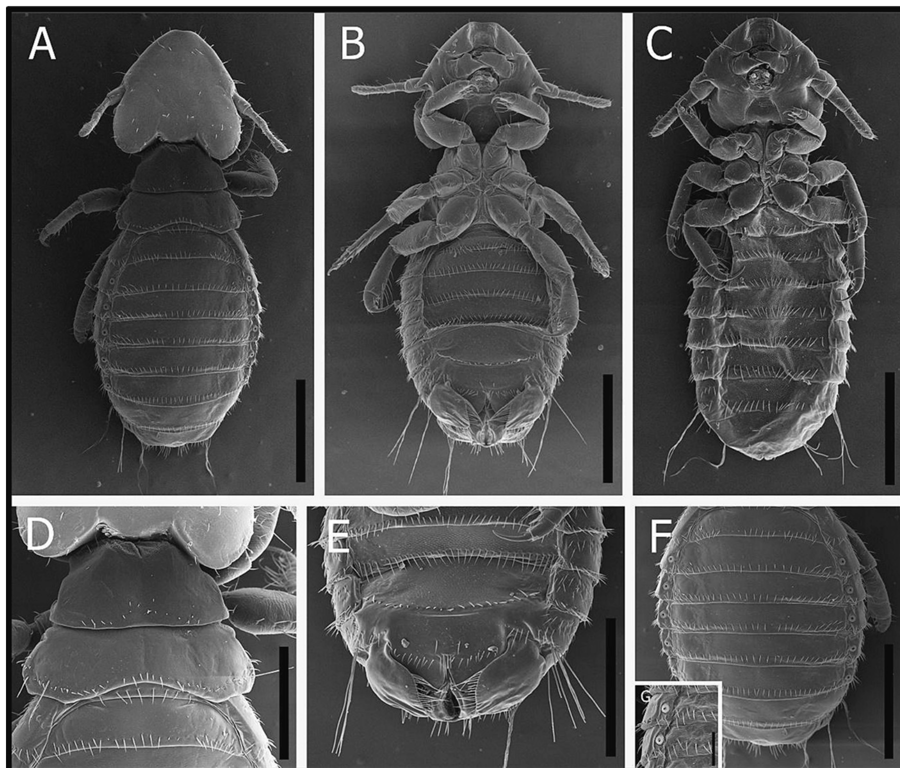

Fig. 1. Scanning electron microscopy images of *Eutrichophilus cordiceps* Mjöberg, 1910. A – Dorsal general view of the female; B – Ventral general view of the female; C – Ventral general view of the male; D – thoracic region of the female prothorax; E – Distal ventral region of the female, focusing the genital area; F – Dorsal view of the female' abdomen; G – Dorsal spiracles. Scale bar: A-C, F 500 μm, D 300 μm, E 400 μm, and G 100 μm.

$4,61 \times 10^0$ to $6,08 \times 10^0$ copies/μL (Mean: $5,34 \times 10^0$ copies/μL). The efficiency (E) ranged from 101.7% to 102.5% (Mean: 102.1%), $R^2 = 0.971$ to 0.989 (Mean: 0.98), Slope ranged from 3.281 to 3.335 (Mean: 3.308), and Intercept (y-int) ranged from 36.581 to 38.013 (Mean: 37.297).

These ten positive samples were subjected to additional PCR assays targeting six molecular markers to characterize the *Bartonella* strain found herein. Only four sequences of the *nuoG* gene were obtained, with sizes ranging from 367 to 374 bp, conforming into two haplotypes. The sequences were deposited in the GenBank database (accession numbers OR593986 to OR593989). When comparing the sequences available in GenBank, the sequences obtained herein were 99.16% (e-value: 2e-180 and 3e-179) identical to *Bartonella* sp. (ON376903), detected in the tick species *Amblyomma sculptum* Berlese, 1888 (Parasitiformes: Ixodida) collected from parasitizing wild boars, *Sus scrofa* Linnaeus, 1758 (Cetartiodactyla: Suidae) in the state of São Paulo. The *rpoB*, *pap31*, *gltA*, *groEL,* and *ftsZ* genes were negative for all ten samples.

3.3. Phylogenetic analysis

The Maximum Likelihood phylogenetic analysis (Fig. 3) based on a 368 bp fragment of the *nuoG* gene implemented with TPM3 + I + G evolutionary model positioned the sequences detected in *E. cordiceps* into the *B. machadoae* clade, closest to *Bartonella* sp. detected in *A. sculptum* collected from wild boars (*Sus scrofa* L., 1758) as said above, with a bootstrap of 100%.

3.4. Haplotype network

According to the phylogenetic analysis, we used eight closely related sequences for the Haplotype Network analysis based on the *nuoG* gene. As a result, four genotypes were identified (Fig. 4). Genotype #1 included a sequence of *B. machadoae* isolated from the echimyid rodent, *Thrichomys fosteri* Thomas, 1903 (Rodentia: Echimyidae) in the Brazilian Pantanal (Mato Grosso do Sul State). Genotype #2 included four sequences, two of them comprising *Bartonella* sequences detected in the tick species *A. sculptum* collected from wild boars, *S. scrofa* in the state of São Paulo, and two of the sequences obtained in the present study, both from *Eutrichophilus cordiceps* collected in Bauru Municipality, São Paulo State. Genotype #3 comprised a single sequence from *Bartonella* sp. also detected in *A. sculptum* parasitizing wild boar in São Paulo State. Genotype #4 included two sequences of *Bartonella* sp., also obtained in the present study, both from the specimens of *E. cordiceps* collected in Lages Municipality, Santa Catarina State.

Data analysis: Nucleotide diversity (π): 0.00469, ±0.0000027; Haplotype diversity (Hd): 0.7500, ±0.01936; Number of variable sites: 6; Average number of nucleotide differences (K): 1.67857; and Percentage of G + C obtained: 40,7%.

4. Discussion

Eutrichophilus cordiceps was reported in Brazil on the Spiny Tree Porcupine, *Coendou spinosus* (F. Cuvier, 1823) (Rodentia: Erethizontidae), in Colônia de Santa Cruz, Paranaguá Municipality, Rio Grande do Sul State, and Nova Teutônia Municipality, Santa Catarina State. Herein, we reported the occurrence of this louse species in São Paulo State (Bauru Municipality) for the first time, and in Santa Catarina State (Lages Municipality) for the second time. Also, in order to help future identifications, we provide SEM images of the male and females, highlighting the diagnostic characters, as well as additional microscopy images of the egg, which had not been described in detail in previous studies. As this is the first time that eggs were characterized in this genus, it is necessary to examine eggs of other species of *Eutrichophilus*, aiming at checking if the ornamentations on the surface of the eggs can be diagnostic characteristics for the genus or species.

Proportionally to the size of the infested porcupine, the zookeepers who collected *E. cordiceps* observed high infestation. Similarly, Werneck (1936) pointed out that lice are able to develop and reproduce more easily in porcupines, since these animals are unable to clean themselves ('grooming') due their spines.

According to the knowledge available in the literature, this is the first time that the genus *Eutrichophilus* has been subject to DNA extraction and successfully retrieved genetic sequences. The obtained 18S rRNA will contribute to future studies regarding phylogenetic inferences to unravel the evolutionary aspects of lice species as well as to aid in taxonomic issues related to this genus.

Bartonella species are highly adapted to mammal reservoirs and associated vectors, e.g., sandflies, human body lice, fleas including the cat flea, biting flies, and ticks [48]. According to the International Code of Nomenclature of Prokaryotes (ICNP), few species have been described directly from an arthropod. For instance, *Bartonella apis* was isolated and described from honeybee midguts [23]. *Bartonella senegalensis* was isolated and described from the tick species *Ornithodoros sonrai* Sautet & Witkowski, 1943 (Acari: Argasidae) [29]. '*Candidatus* Bartonella cariotis' was first detected in the human biting kissing bug species, *Eratyrus mucronatus* (Stål, 1859) (Reduviidae: Triatominae) [24,33].

Regarding lice-*Bartonella* association, the human body lice, *Pediculus humanus* Linnaeus, 1758 (Anoplura: Pediculidae), has been pointed out as the main vector of *Bartonella quintana* [5]. Additionally, *Bartonella* spp. have been molecularly detected in other louse species, such as *Haematopinus quadripertussus* Fahrenholz, 1916 (Anoplura: Haematopinidae) parasitizing cattle, *Bos taurus* Linnaeus, 1758 (Artiodactyla: Bovidae) in Israel [17], in *Hoplopleura sciuricola* Ferris, 1921 (Anoplura: Hoplopleuridae) and *Neohaematopinus sciuri* Jancke, 1932 (Anoplura: Polyplacidae) parasitizing the Eastern Gray Squirrel, *Sciurus carolinensis* Gmelin, 1788 (Rodentia: Sciuridae), in Georgia, USA [13], and in *N. sciuri* parasitizing *S. carolinensis* in South Carolina, USA [31].

For the Neotropical Region, *Bartonella mellophagi* was detected in pools of the louse species *Bovicola ovis* (L., 1758) (Ischnocera: Trichodectidae) parasitizing sheep, and *Bartonella capreoli* in pools of *Linognathus africanus* Kellogg and Paine, 1911 (Anoplura: Linognathidae) parasitizing goats in Mexico [3]. In Peru, the species *B. bovis* was detected again with *Bartonella* sp. [15]. While in Brazil, *Bartonella* DNA was detected in a pool of the louse species *Felicola subrostratus* Burmeister, 1838 (Ischnocera: Trichodectidae) parasitizing domesticated cats [38].

In the present study, we were able to detect, for the first time, *Bartonella* sp. in *E. cordiceps* from two different localities in Brazil. The sequences obtained showed to be closely related to *Bartonella machadoae,* recently isolated and fully characterized from wild rodents in the Brazilian Pantanal [12], as well to *Bartonella* sequences previously detected in ticks, *A. sculptum* in southeastern Brazil [42]. The haplotype network based on a fragment of the *nuoG* gene showed that the sequences obtained in the present study from *E. cordiceps* of Santa Catarina State (genotype #4) differ from the others due to at least one mutational event. The sequences obtained from this louse species from São Paulo State (genotype #2) belong to the same genotype detected in ticks from the Pantanal region of Brazil. These genotypes differ by at least three mutational events from the *B. machadoae*.

Finally, the present study showed, for the first time, that chewing lice can also harbor *Bartonella*. Unfortunately, we could not examine or obtain blood samples from the Spiny Tree Porcupines studied. These new associations between *Bartonella* and chewing lice might indicate that these lice species could be responsible for maintaining this bacterium in this mammal, or simply these genotypes found herein are lice endosymbionts. Unfortunately, due to the low number of copies of this agent in the lice studied, we could only obtain sequences for the *nuoG* gene. Therefore, more in-depth studies must be carried out to characterize these *Bartonella* strains, to better understand the louse-pathogen-host relationship, and to draw more robust phylogenetic inferences with other *Bartonella* spp.

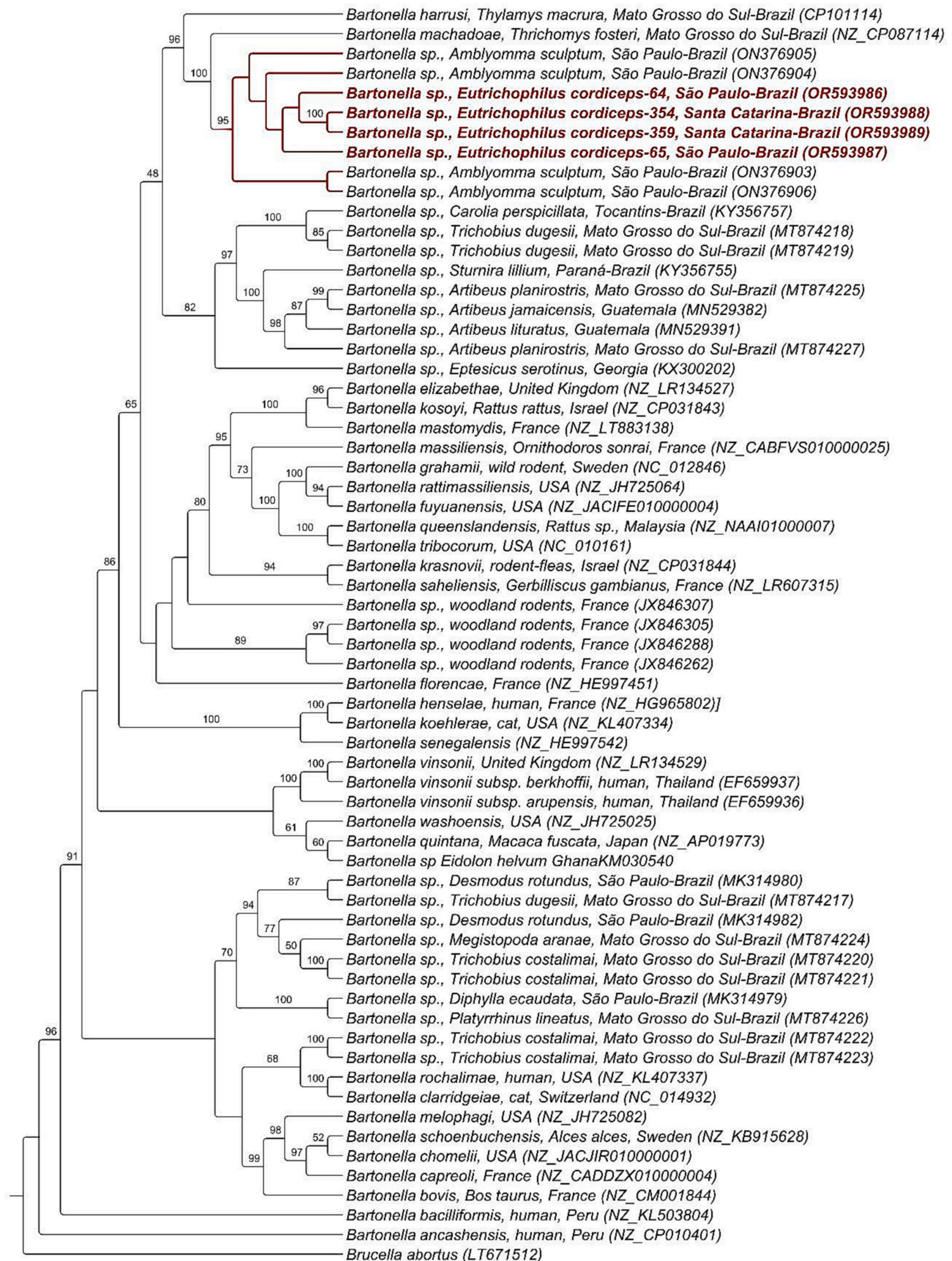

Fig. 3. Phylogenetic analysis of the *Bartonella nuoG* sequences. An approximately 380-bp alignment was generated with TPM3 + I + G evolutionary model, by Maximum Likelihood analysis. *Brucella abortus* (Rhizobiales: Brucellaceae) was used as outgroup.

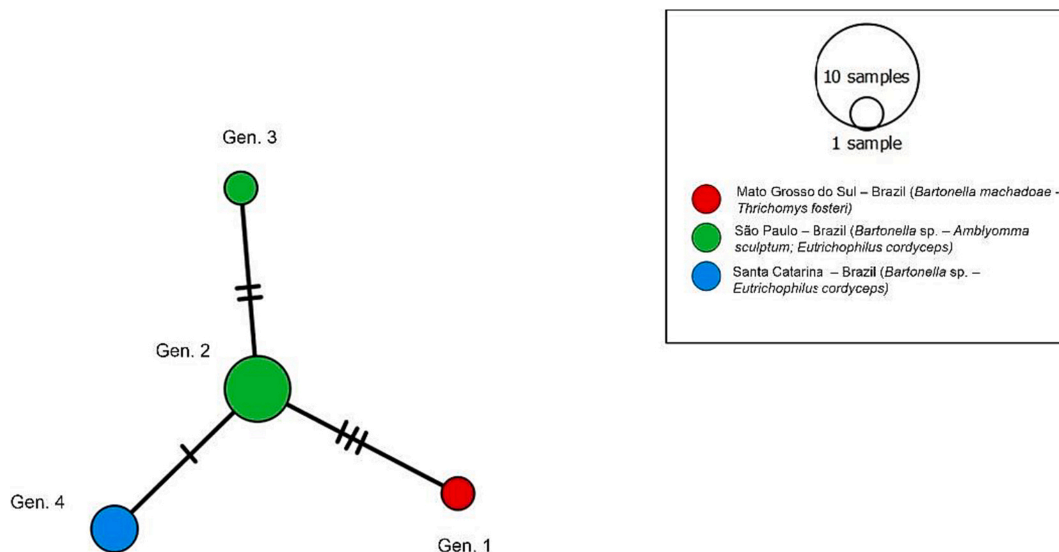

Fig. 4. Haplotype network constructed with *nuoG* gene sequences of *Bartonella* close related with the sequences obtained in the present study. The size of the circles varies according to the number of sequences belonging to each haplotype. While, the colors represent Brazilian State localities, and the black linear stroke lines represent mutational events between each haplotype.

CRediT authorship contribution statement

Ricardo Bassini-Silva: Writing – review & editing, Writing – original draft, Validation, Methodology, Data curation, Conceptualization. **Maria Eduarda Moraes das Chagas:** Writing – original draft, Methodology, Data curation. **Victor de Souza Mello-Oliveira:** Writing – original draft, Methodology, Data curation, Conceptualization. **Ana Cláudia Calchi:** Validation, Methodology, Formal analysis, Data curation. **Ana Carolina Castro-Santiago:** Methodology, Data curation, Conceptualization. **Lívia de Oliveira Andrade:** Validation, Resources, Methodology, Data curation. **Gabriela Coelho Benedet:** Validation, Methodology, Data curation. **Fernanda Mara Aragão Macedo Pereira:** Methodology, Investigation, Conceptualization. **Lauro Leite Soares-Neto:** Methodology, Data curation. **Alícia Giolo Hippólito:** Resources, Methodology, Data curation. **Estevam Guilherme Lux Hoppe:** Writing – review & editing, Supervision, Funding acquisition, Data curation. **Karin Werther:** Writing – review & editing, Supervision, Funding acquisition, Data curation. **Marcos Rogério André:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Rosiléia Marinho de Quadros:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition. **Darci Moraes Barros-Battesti:** Writing – review & editing, Supervision, Investigation, Data curation. **Sebastián Muñoz-Leal:** Writing – review & editing, Supervision, Investigation, Data curation. **Fernando de Castro Jacinavicius:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

The data supporting the findings of this study are available within the article. As well as all material is in their respective collections and are available for public consultation.

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