

Molecular characterization of ‘*Candidatus Borrelia tachyglossi*’ (family *Spirochaetaceae*) in echidna ticks, *Bothriocroton concolor*

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Abstract

Recently, a novel species of the genus *Borrelia* was identified in *Bothriocroton concolor* and *Ixodes holocyclus* ticks from echidnas. Analyses of 16S rRNA and *flaB* genes identified three closely related genotypes of this bacterium (*Borrelia* sp. Aus A–C) that were unique and distinct from previously described borreliae. Phylogenetic analyses of *flaB* (763 bp), *groEL* (1537 bp), *gyrB* (1702 bp) and *glpQ* (874 bp) gene sequences and concatenated sequences (3585 bp) of three gene loci (16S rRNA, *flaB* and *gyrB*) were consistent with previous findings and confirm that this novel species of the genus *Borrelia* is more closely related to, yet distinct from, the Reptile-associated (REP) and Relapsing Fever (RF) groups. At the *flaB* locus, genotypes A, B and C shared the highest percentage sequence similarities (87.9, 88 and 87.9%, respectively) with *B. orrelia turcica* (REP), whereas at the *groEL* and *gyrB* loci, these genotypes were most similar (88.2–89.4%) to *B. orrelia hermsii* (RF). At the *glpQ* locus, genotypes A and B were most similar (85.7 and 85.4% respectively) to *Borrelia* sp. Tortoise14H1 (REP). The presence of the *glpQ* gene, which is absent in the Lyme Borreliosis group spirochaetes, further emphasises that the novel species of the genus *Borrelia* characterized in the present study does not belong to this group. Phylogenetic analyses at multiple loci produced consistent topographies revealing the monophyletic grouping of this bacterium, therefore providing strong support for its species status. We propose the name ‘*Candidatus Borrelia tachyglossi*’, and hypothesize that this species of the genus *Borrelia* may be endemic to Australia. The pathogenic potential of this bacterium is not yet known.

The family *Spirochaetaceae* is classified under the order *Spirochaetales*, belonging to the phylum *Spirochaetes*. This family consists of genera that are of concern to human health, such as *Borrelia* and *Treponema* [1], with common pathogenic species including ‘*Treponema pallidum*’ subsp. *pallidum*, the causative agent of syphilis worldwide [2], and ‘*Treponema pallidum*’ subsp. *pertenue*, the bacterium responsible for yaws [3]. The genus *Borrelia* is a member of the family *Spirochaetaceae* and through convention is divided into three major clades: Lyme disease/Borreliosis (LB) caused by members of the *Borrelia burgdorferi sensu lato* complex, the Relapsing Fever (RF) borreliae and the Reptile-associated (REP) borreliae [4]. The LB borreliae currently comprise over 20 recognized species including the primary Northern hemisphere Lyme-disease-causing agents *Borrelia afzelii*, *Borrelia bavariensis*, *Borrelia burgdorferi sensu stricto*, and *Borrelia garinii*, along with a newly described genospecies ‘*Candidatus Borrelia mayonii*’ that causes LB in the upper Midwestern USA [5–8]. Members of the LB group are vectored by hard ticks (family Ixodidae), with the pathogenic species commonly transmitted to

humans and other animals by ticks within the *Ixodes ricinus* complex: *Ixodes ricinus* in Europe, *Ixodes persulcatus* in Europe and Asia, and *Ixodes pacificus* and *Ixodes scapularis* in USA [7]. These pathogenic spirochaetes are dependent on wildlife, particularly rodents and birds, which act as asymptomatic reservoir hosts that maintain their life cycles and transmission [9].

Spirochaetes within the RF group have been reported throughout a number of continents, including Africa [10], Eurasia [11] and North America [12]. These borreliae, in contrast to the LB group, are generally transmitted by soft ticks (family Argasidae), with the exceptions of *Borrelia miyamotoi* identified in *I. persulcatus* [13], *I. ricinus* [14], *I. pacificus* [15] and *I. scapularis* [16]; ‘*Borrelia lonestari*’ in *Amblyomma americanum* [17]; *Borrelia theileri* in *Rhipicephalus microplus* [18]; and ‘*Candidatus Borrelia texasensis*’ in *Dermacentor variabilis* [19].

The third major clade of this genus, the REP borreliae, was identified after the discovery of *Borrelia turcica* in *Hyalomma aegyptium* ticks collected from tortoises in Turkey

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Abbreviations: LB, Lyme disease/Borreliosis; REP, Reptile-associated; RF, Relapsing Fever.

All sequences generated in this study were deposited in GenBank/EMBL/DBJ under accession numbers KY586964–KY586974.

One supplementary figure and seven supplementary tables are available with the online Supplementary Material.

[20, 21], followed by subsequent addition of REP-related species of the genus *Borrelia* identified in various reptiles [4, 22]. While the LB and RF spirochaetes consist of zoonotic pathogens and are of significant public health concern in many countries, the pathogenicity and zoonotic potential of the REP group are not yet known.

Although LB borreliae have never been identified in Australian ticks, wildlife or people [23], two RF borreliae are recognized: *B. theileri* and *Borrelia anserina* that are transmitted by *Rhipicephalus (Boophilus) australis* [24, 25] and *Argas persicus* [26, 27], respectively. In addition, '*Borrelia queenslandica*' was the first species of the genus *Borrelia* to be reported from native long-haired rats, *Rattus villosissimus*, in north-west Queensland, Australia. While the soft tick, *Ornithodoros gurneyi*, was considered to be the vector of this species due to its presence in the region, transmission experiments were not successful, and molecular characterization was never conducted to reliably identify the species of the genus *Borrelia* [28, 29].

Sequence analysis of multiple loci offers the advantage over morphological characterization of being highly discriminatory, therefore serving as a reliable method for accurate identification, characterization and population, and epidemiological analyses in numerous bacterial studies [30, 31]. This technique was first used on *Borrelia burgdorferi* in 2008 [32] and has become an increasingly common technique for taxonomic and epidemiological studies of this genus [33–36].

Recently, a novel species of the genus *Borrelia* was detected in a number of echidna ticks, *Bothriocroton concolor*, collected [37]. An additional representative was detected in an *Ixodes holocyclus* tick [38]. Based on the 16S rRNA (1097 bp) and flagellin (*flaB*, 400 bp) gene phylogenetic analyses, this species of the genus *Borrelia* formed a distinct clade from other well-described borreliae, indicating this organism, designated '*Borrelia* sp. Aus', to be unique [37]. In the present study, we conducted sequence analyses of the *flaB*, *groEL*, *glpQ* and *gyrB* genes, in addition to the 16S rRNA and short *flaB* loci reported previously [37], to study the relationship of this novel species to other of the genus *Borrelia*. Phylogenetic analysis confirmed its species status, and we hereby propose to designate this species as '*Candidatus Borrelia tachyglossi*'.

This study was conducted under the compliance of the *Australian Code for the Responsible Conduct of Research, 2007* and *Australian Code for the Care and Use of Animals for Scientific Purposes, 2013*. Tick collection was carried out opportunistically with the approval from the Murdoch University Animal Ethics Committee.

Genomic DNA was extracted previously from 97 *Bothriocroton concolor* ticks, with 38 (39%) ticks testing positive at the *Borrelia*-specific *flaB* locus [37]. These 38 *Borrelia*-positive ticks were included in this study. Nested- and hemi-nested PCRs were conducted using primers targeting the housekeeping genes *flaB* (763 bp), *groEL* (1537 bp), *glpQ*

(874 bp) and *gyrB* (1702 bp) (Table S1, available in the online Supplementary Material). Primers were designed to amplify short fragments of each gene with overlapping regions in order to obtain maximum coverage of the genes analysed for accurate characterization. PCR cycling conditions were as described by Loh et al. [37] (see Table S1 for respective annealing temperatures), with the exception of *groEL* primers: initial denaturation at 95 °C for 5 mins, 35 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 40 s, extension at 72 °C for 2 mins, and a final extension at 72 °C for 7 mins. Amplification products of the targeted DNA products were electrophoresed in 1–2% agarose gel with blue light using safe (Invitrogen), and positive samples were purified and sent for Sanger sequencing.

DNA sequences generated at *flaB*, *groEL*, *glpQ* and *gyrB* loci were aligned and analysed together with sequences representing species of the genus *Borrelia* retrieved from GenBank. All sequences were aligned using MAFFT v7.017 [39] and then refined using MUSCLE [40]. The best-fit model for each locus was assessed using MEGA6 [41] and was selected based on the Bayesian Information Criterion (BIC). Bayesian phylogenetic reconstructions using sequence alignments of all four loci were generated using MrBayes 3.2.6 [42], and concatenated phylogenetic reconstructions using concatenated sequence alignments were produced using the CIPRES Science Gateway V.3.3 [43]. GTR and HKY substitution models were selected, with gamma categories of five, MCMC length of 1 100 000, burn-in length of 10 000 and subsampling frequency of 200.

In the present study, *Borrelia*-specific *flaB* (763 bp), *groEL* (1537 bp), *gyrB* (1702 bp) and *glpQ* (874 bp) DNA sequences were successfully amplified and sequenced from 38 *Borrelia*-positive *Bothriocroton concolor* ticks described by Loh et al. [37]. Thirty samples were positive for all *flaB* fragments; 24 samples were positive for all *glpQ* fragments; 13 samples were positive for all *groEL* fragments; and 10 samples were positive for all *gyrB* fragments.

Previously, three closely related genotypes were distinguished in the *Bothriocroton concolor* ticks using the 16S rRNA gene sequences, tentatively given the designations *Borrelia* sp. Aus A, *Borrelia* sp. Aus B and *Borrelia* sp. Aus C [37]. However, in the present study, these genotypes are referred as '*Candidatus Borrelia tachyglossi*' genotypes A, B and C, respectively. At the *flaB* locus, genotypes A, B and C consisted of nine, three and five identical samples, respectively. The *flaB* gene alignment (787 bp) between '*Candidatus Borrelia tachyglossi*' genotypes and other described species of the genus *Borrelia* showed that the novel genotypes shared highest percentage sequence identities with *Borrelia turcica* from the REP group (87.9–88%); similarities to the LB *Borrelia* group ranged from 82.1–83.2%; with the least similarity to *Borrelia hispanica* from the RF group (79.8–80%). The percentage identities within the '*Candidatus Borrelia tachyglossi*' genotypes ranged from 99.6–99.9%. The percentage nucleotide identities between '*Candidatus Borrelia tachyglossi*' genotypes and *Borrelia turcica* (87.9–

88 %) were higher than that between *Borrelia turcica* and *Borrelia hermsii* (86.7 %). In contrast, the percentage nucleotide identities between ‘*Candidatus Borrelia tachyglossi*’ genotypes and *Borrelia hermsii* (84.6–84.8 %) were lower than that between *Borrelia turcica* and *Borrelia hermsii* (86.7 %) (Table S2). Phylogenetic analyses of the *flaB* gene locus showed that the ‘*Candidatus Borrelia tachyglossi*’ genotypes clustered most closely with *Borrelia turcica* with a high posterior probability (Fig. 1).

At the *glpQ* locus, genotypes A and B consisted of eight and two identical samples respectively. The amplification of this gene was not successful for genotype C. The *glpQ* nucleotide alignment (947 bp) between ‘*Candidatus Borrelia tachyglossi*’ genotypes exhibited 83.9–85.7 % similarity with the REP species of the genus *Borrelia* and lower similarities (80.6–84.8 %) with the RF species of the genus *Borrelia*. The percentage similarity within ‘*Candidatus Borrelia tachyglossi*’ genotypes was 98.6 %. Phylogenetic analysis confirmed the closer relationship of the ‘*Candidatus Borrelia tachyglossi*’ genotypes from the present study with the REP *Borrelia* group (100 % bootstrap support) (Fig. 1). The percentage nucleotide identities between ‘*Candidatus Borrelia tachyglossi*’ genotypes and *Borrelia turcica* (84.1–84.2 %) and *Borrelia coriaceae* (RF) (84.1–84.8 %) were higher than that between *Borrelia turcica* and *Borrelia coriaceae* (83.3 %) (Table S3).

At the *groEL* locus, ‘*Candidatus Borrelia tachyglossi*’ genotypes A and B were identical, and both shared 99.9 % similarities with ‘*Candidatus Borrelia tachyglossi*’ genotype C. Nucleotide alignment (1540 bp) between ‘*Candidatus Borrelia tachyglossi*’ genotypes and other described species of the genus *Borrelia* showed that the novel isolates had the least similarity with the LB group (83.9–85 %) and were most similar to *Borrelia hermsii* from the RF group (89.3–89.4 %). Phylogenetic analysis of the *groEL* locus showed that ‘*Candidatus Borrelia tachyglossi*’ genotypes from the present study clustered with the RF group with strong posterior probability support (Fig. 1). The percentage nucleotide identities between ‘*Candidatus Borrelia tachyglossi*’ genotypes and *Borrelia hermsii* (89.3–89.4 %) were higher than that between *Borrelia hermsii* and *Borrelia burgdorferi* (84.8 %); whereas the percentage identity between ‘*Candidatus Borrelia tachyglossi*’ genotypes and *Borrelia burgdorferi* (84.3 %) was slightly lower (Table S4). The genetic distance between ‘*Candidatus Borrelia tachyglossi*’ and *Borrelia turcica* at this locus remains to be determined until the *groEL* gene is characterized in *Borrelia turcica*.

At the *gyrB* locus, ‘*Candidatus Borrelia tachyglossi*’ genotypes A and B were identical. Nucleotide alignment (1712 bp) revealed that the ‘*Candidatus Borrelia tachyglossi*’ genotypes exhibited 80.8–82.0 %, 80.8–82.0 % and 84.8–88.2 % sequence similarity with the LB *Borreliae*, the RF group and *Borrelia turcica*, respectively. The percentage similarities within ‘*Candidatus Borrelia tachyglossi*’ genotypes ranged from 99.6 to 99.9 %. Phylogenetic analysis showed that ‘*Candidatus Borrelia tachyglossi*’ genotypes

formed their own monophyletic clade, separate from *Borrelia turcica* and the RF group, with high bootstrap support (98 %) (Fig. 1). The percentage nucleotide identities between ‘*Candidatus Borrelia tachyglossi*’ genotypes and *Borrelia turcica* (87.8–88 %) and *Borrelia hermsii* (88.1–88.2 %) were higher than that between *Borrelia turcica* and *Borrelia hermsii* (86.7 %) (Table S5).

A Bayesian phylogenetic tree reconstructed using the concatenated alignment (3585 bp) consisting of three genes, 16S rRNA, *flaB* and *gyrB*, available for each of the main *Borreliae* (Fig. 1), illustrated that the ‘*Candidatus Borrelia tachyglossi*’ genotypes from *Bothriocroton concolor* ticks grouped separately, with *Borrelia turcica* as the closest relative (91.1–91.2 % nucleotide identities) (Table S6). Likewise, the concatenated alignment (5154 bp), which excluded *Borrelia turcica* (REP), based on the four loci amplified in the present study (16S rRNA, *flaB*, *groEL* and *gyrB*) also produced a similar tree topology with ‘*Candidatus Borrelia tachyglossi*’ genotypes forming a monophyletic clade supported by high posterior probabilities (Fig. S1), sharing the highest percentage identities with *Borrelia hermsii* (90.3 %) (Table S7).

All phylogenetic trees reconstructed revealed similar topologies with the REP group species of the genus *Borrelia* as the closest sister clade. The recently established REP group has been detected in various reptiles from several countries and in ticks that parasitise them [4, 21]. The recent discovery of a novel species of the genus *Borrelia* in *Amblyomma varenense* collected from the reticulated python (*Python reticulatus*) showed that the species of the genus *Borrelia* identified clustered together with the REP-associated *Borrelia* group, along with *Borrelia turcica*, based on phylogenetic analyses of 16S rRNA and *flaB* genes [44]. However, the pathogenic potential of the species of the genus *Borrelia* belonging to the REP group is unknown.

A number of members within the genus *Borrelia* are well known to cause diseases in humans outside of Australia; nonetheless, borreliac tick-borne disease in humans still remains highly speculative and controversial in this country. ‘*Candidatus Borrelia tachyglossi*’ was first reported in 2016 [37], hence the urgency to further characterize this bacterium on the basis of multi-loci gene sequencing in order to confirm the taxonomic position of this new member in the genus *Borrelia*. Our results, based on sequence and phylogenetic characterization of multiple loci, provide conclusive evidence that ‘*Candidatus Borrelia tachyglossi*’, identified in *Bothriocroton concolor* ticks from echidnas, is distinct from other described species of the genus *Borrelia* and constitutes to a new clade in this genus.

Borreliac spirochaetes are well known to be associated closely with wildlife and utilize tick vectors to maintain a sylvatic life cycle [45, 46]. Australian wildlife are also known to be involved in spill-over of various zoonotic parasites [47]. Therefore, it is plausible that this bacterium is also likely to persist in the environment through circulation

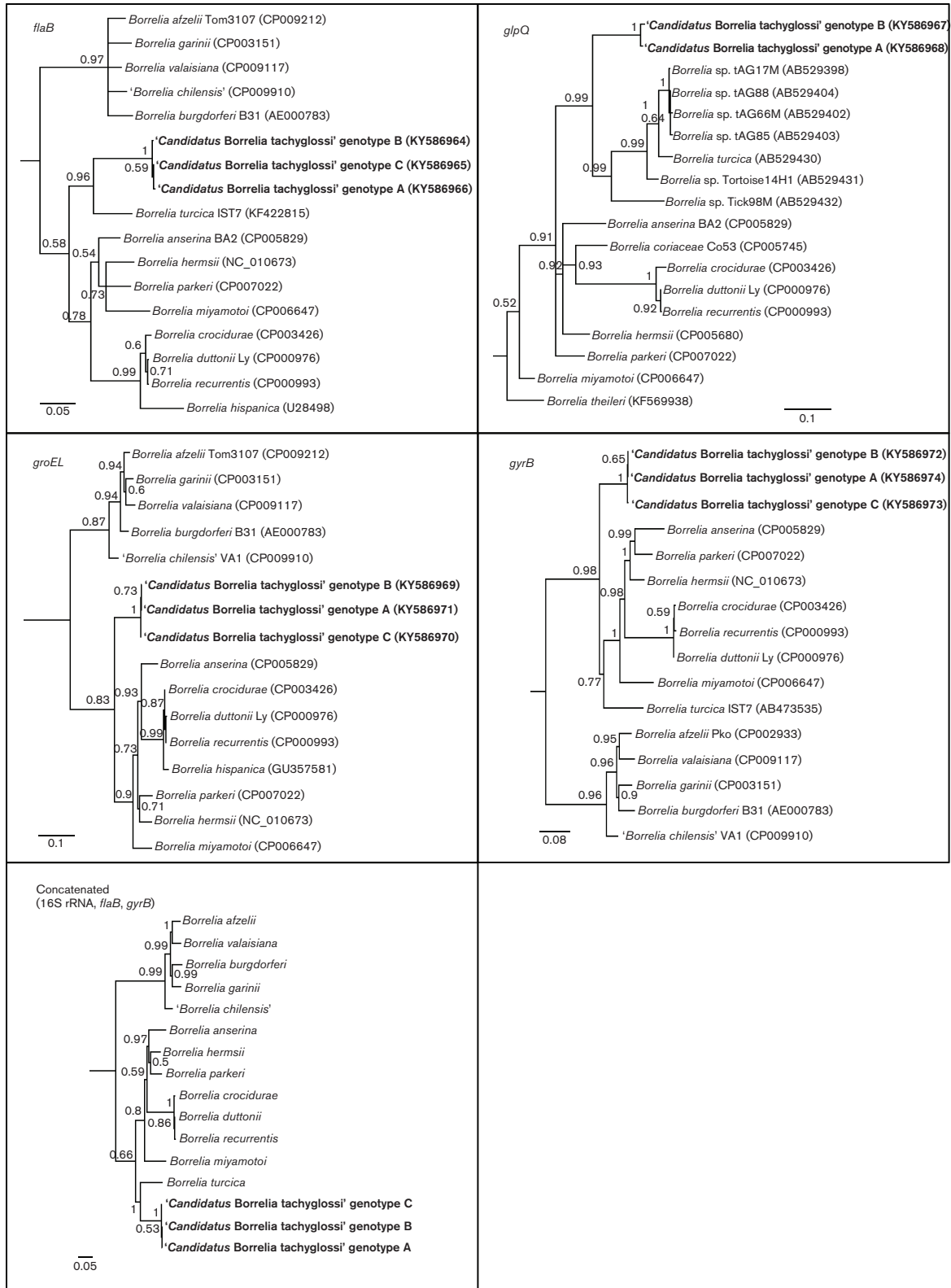


Fig. 1. Phylogenetic reconstructions based on *flaB*, *groEL*, *glpQ* and *gyrB* gene sequences, and concatenated gene sequences of '*Candidatus Borrelia tachyglossi*' genotypes A, B, and C identified in *Bothriocroton concolor* ticks from echidnas. *Brachyspira pilosicoli* (AY241832), *Treponema pallidum* (NZ_CP010566), *Escherichia coli* (X56907) and *Spirochaeta lutea* (JNUP01000064) were used as

outgroups for each gene, respectively. Sequences determined in this study are indicated in bold type. Node labels represent posterior probabilities. Bars, substitutions per nucleotide position.

among native ticks and native mammalian (including marsupial) hosts. Unlike the Australian paralysis tick *I. holocyclus*, *Bothriocroton concolor* is a highly specialized tick, with echidnas as their primary host, and with a geographic distribution known only in Australia and Papua New Guinea [48]. ‘*Candidatus Borrelia tachyglossi*’ has previously been identified in one human-biting tick (*I. holocyclus*) removed from an echidna [38] and its prevalence in *I. holocyclus* or other human-biting ticks remains to be determined. The morphological characteristics and the pathogenicity of this bacterium are also unknown.

DESCRIPTION OF ‘*CANDIDATUS BORRELIA TACHYGLOSSI*’

‘*Candidatus Borrelia tachyglossi*’ (ta.chy.glos’si. N.L. gen. n. *tachyglossi* of *Tachyglossus aculeatus*, the monotreme host of the ticks in which the bacterium was first identified)].

Species can be differentiated from other borreliae based on sequence and phylogenetic analyses of five genomic loci (16S rRNA, *flaB*, *groEL*, *gyrB* and *glpQ*). Comparisons of the *flaB* gene sequences among the ‘*Candidatus Borrelia tachyglossi*’ genotypes showed two single nucleotide polymorphisms (SNPs) in genotype A at bases 485 and 519 (GenBank accession no. KY586966); and one SNP in genotype B at base 227 (KY586964). As for the *groEL* gene, analysis revealed two SNPs in genotype C at bases 656 and 1143 (KY586970). Analysis of the *gyrB* gene showed two SNPs in genotype B at bases 985 and 1416 (KY586972); and five SNPs in genotype C at bases 405, 757, 1075, 1093 and 1420 (KY586973). At the *glpQ* locus, genotype A (KY586968) and genotype B (KY586967) showed 12 base differences at bases 3, 43, 103, 112, 264, 515, 520, 530, 532, 702, 819 and 852.

The DNA G+C contents for 16S rRNA, *flaB*, *groEL*, *gyrB* and *glpQ* genes of ‘*Candidatus Borrelia tachyglossi*’ genotype A are 47.3, 40.9, 38.5, 33.8 and 34.1 mol%, respectively. The DA G+C contents for 16S rRNA, *flaB*, *groEL*, *gyrB* and *glpQ* genes of ‘*Candidatus Borrelia tachyglossi*’ genotype B are 47.2, 40.9, 38.5, 33.9 and 34.7 mol%, respectively. The DNA G+C contents for 16S rRNA, *flaB*, *groEL*, and *gyrB* genes of ‘*Candidatus Borrelia tachyglossi*’ genotype C are 47.2, 41, 38.5 and 34 mol%, respectively.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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