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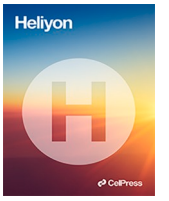

Research article

# Molecular prevalence of *Bartonella* spp. in bat flies in east coast Malaysia

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A B S T R A C T

Bats are a significant reservoir for numerous pathogens, including *Bartonella* spp. It is one of the emerging zoonotic bacterial diseases that can be transmitted to humans and may cause various unspecific clinical manifestations. Thus, bartonellosis is rarely diagnosed and is regarded as a neglected vector-borne disease (VBD). Bat flies have been hypothesised to be a vector in the transmission of pathogens among bats. They are host-specific, which reduces the likelihood of pathogen transmission across bat species; however, they are likely to maintain high pathogen loads within their host species. To explore the presence of *Bartonella* spp. in bat flies from Peninsular Malaysia; bat fly samples collected from various sites at the east coast states were subjected to molecular detection for *Bartonella* spp. It was discovered that 38.7 % of bats from Terengganu and Kelantan were infested with bat flies; however, no bat fly was found in bats collected from Pahang. The collected bat flies belonged to the families Nycteribiidae (79.6 %) and Streblidae (20.4 %). The collected bat flies were pooled according to the locations and species into 39 pools. Out of these 39 pools, 66.7 % (n = 26) were positive for *Bartonella* spp. by PCR. Sequence analyses of five randomly selected PCR-positive pools revealed that pools from Kelantan (n = 3) have the closest sequence identities (99 %) to *Bartonella* spp. strain Lisso-Nig-922 from Nigeria. However, the other pools from Terengganu (n = 2) were closely related to *Bartonella* spp. strain KP277 from Thailand and *Bartonella* spp. strain Rhin-3 from the Republic of Georgia with 99 % and 100 % sequence identity, respectively. This suggests that the *Bartonella* spp. found in Malaysian bat flies are genetically diverse and can potentially serve as reservoirs for pathogenic *Bartonella* spp.

## 1. Introduction

According to the data retrieved from globalforestwatch.org in 2023, Malaysia was ranked 9th among the world's highest rate of

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forest loss countries between 2001 and 2021. Peninsular Malaysia has a long history of converting natural forests to agricultural/commodity plantations, especially for oil palm [1]. Deforestation, disturbance, and habitat loss have threatened Malaysian biodiversity [2] and increased human-wildlife conflict or coexistence [3]. This also affects wildlife deep in the forests, including bats [4,5].

Bats are one of the most abundant mammals in the tropical rainforests of Malaysia; they account for 40 % of overall mammals in Malaysia [6,7]. Due to their feeding habits, bats play important roles as pollinators, seed dispersers, and forest insect regulators [8,9]. Despite all the benefits of bats, they are also recognised as a significant reservoir for numerous pathogens that can be transmitted to humans [10,11]. There is a growing list of recent bat-borne zoonotic spillover events through domesticated animal hosts, such as horses and pigs, if not directly via direct contact with bats by intruding into bat roosting caves for ecotourism or hunting purposes [12].

Bartonellosis is one of the emerging zoonotic bacterial diseases responsible for various human clinical syndromes that bats can transmit [13]. *Bartonella* spp. is a fastidious, gram-negative, and intracellular haemotropic bacteria. More than 114 *Bartonella* species have been identified (https://lpsn.dsmz.de/search?word=bartonella), many of which are zoonotic pathogens. It can spread among bat populations through hematophagous arthropods such as flies, fleas, lice, and ticks [14]. Among the bat ectoparasites, bat flies (Diptera: Nycteribiidae and Streblidae) are one of the common potential vectors in transmitting and maintaining *Bartonella* spp. in bat populations [15].

Bat flies are highly host-specific ectoparasites living in the fur and on wing membranes of the bat host [16,17]9. Although they are host-specific, the obligation of bat flies on bats may play a vital role in maintaining the *Bartonella* spp. among bat populations. Reports on Malaysian bat flies and their associated pathogens are limited [14], with only two recent studies reporting on the detection of *Bartonella* in *Pteropus hypomelanus* [18] and *Cynopterus brachyotis* [19]2). This renders many possibilities for revealing the diversity and association of *Bartonella*, bat flies, and bats in this region and enriches our understanding of the evolutionary history of this triad globally [20]. Considering the overall scarcity of *Bartonella* studies in bats from Southeast Asia, especially from Malaysia, the present study aims to determine the prevalence of *Bartonella* spp. in bat flies from various bat species at the east coast of Peninsular Malaysia.

## 2. Materials and methods

Bat flies were collected previously during field excursions from three localities (Terengganu, Pahang and Kelantan) at the east coast of Peninsular Malaysia from August to October 2021, and their records were used in this study. Bats were trapped using mist nets, and the bat species were determined directly in the field before being released. The collected bat flies were carefully removed from the hosts using fine forceps, preserved in 90 % ethanol, and stored at −80 °C for further laboratory identification and polymerase chain reaction (PCR) procedures. Bat species identification was performed using morphometric measurements, and morphological features were compared to a reference by Francis [21] and Kingston [8]. No anaesthetic or immobilisation agents were used during bat capture or handling. Bat flies were identified based on the publications of Jobling [22–25], Theodor [26], and Maa [27–30]. All the procedures on bats and bat fly sampling were reviewed and approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Malaysia (UMK/FPV/ACUC/PG/6/2021).

Bat flies were then pooled according to the locality, bat species and bat fly species before being subjected to PCR. In some instances, bat flies obtained from different hosts but belonging to the same species were pooled together. A total of 39 pools with 2–4 individual bat flies in a pool were tested for *Bartonella* spp. The samples were triturated and manually homogenised using a plastic pestle in a micro-centrifuge tube. The DNA was extracted using a Genomic DNA Mini Kit (Tissue) (Geneaids, Taiwan) according to the recommended manufacturer procedures. Primers for amplification of the *Bartonella* 16S–23S ribosomal RNA intergenic spacer (ITS) (ITS325_F - 5′-CTT CAG ATG ATG ATC CCA AGC CTT TTG GCG-3′ and ITS1100_R - 5′-GAA CCG ACG ACC CCC TGC TTG CAA AGC-3′) and the *Bartonella* citrate synthase gene (*gltA*) (BhCS.781p - 5′-GGG GAC CAG CTC ATG GTG G-3′ and BhCS.1137n - 5′-AAT GCA AAA AGA ACA GTA AAC A-3′) that produce expected products of 408–717bp and ~380bp respectively, were used in the present study [31,32]. The PCR conditions included an initial denaturation step at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 66 °C for 1 min, extension at 72 °C for 1 min and a final extension step at 72 °C for 5 min. PCR-positive products from the bat fly pools were sequenced, and the obtained sequences were analysed using ChromasPro software (Technelysium Pty Ltd, Australia). The obtained nucleotide sequences were compared with sequences in the GenBank of the National Centre for Biotechnological Information (NCBI - www.ncbi.nim.nih.gov) using the Basic Local Alignment Search Tool (BLAST) programme. A unidirectional workflow and strict laboratory protocols were instituted to prevent the potential contamination of reagents and samples. Nuclease-free water and *B. henselae* Houston-1; ATCC 49882 were incorporated into every PCR run as negative and positive controls, respectively, to maintain the integrity of the PCR assays.

A phylogenetic tree was constructed by aligning the sequences using ClustalX 2.1 program [33] and neighbor-joining software [34] with bootstrap values of 100 replicates. The tree was plotted based on the partial 16S–23S ribosomal RNA ITS gene sequences of five *Bartonella* spp. of this study and compared with 16 reference sequences of *Bartonella* spp. from the NCBI-GenBank. *Burkholderia pseudomallei* from Malaysia (Accession no. FJ981704) was used as an outgroup.

## 3. Results

A total of 186 bats (15 species) were captured, comprising 103 bats (9 species) from Gunung Reng, Kelantan, 53 bats (9 species) from Sekayu Recreational Forest, Terengganu, and 30 bats (4 species) from the Pahang National Park, Merapoh, Pahang. The number of bat species in Kelantan was similar to Terengganu, in which nine species were sampled and recorded. Pahang had the least bat species sampled.

As documented in Table 1, 38.7 % (72/186) of bat species were infested with bat flies. Almost half (49.5 %) of the bats from

Kelantan were infested with bat flies, followed by bats from Terengganu (35.9 %), while none from Pahang were infested with bat flies. At Gunung Reng, Kelantan, the bat species with the highest bat fly infestation was *Eonycteris spelaea* 66.7 % (38/57), followed by *Rhinolophus pusillus* 50.0 % (1/2), *Rhinolophus affinis* 43.8 % (14/32) and *R. stheno* 25.0 % (1/4). The other bat species, such as *Hipposideros dyacorum*, *H. larvatus*, *H. kunzi*, *Taphozous melanopogon*, and *Myositis muricola* were negative for bat fly infestation. For the bat species from Sekayu Recreational Forest, Terengganu, *R. affinis* 52.6 % (10/19) had the highest bat fly infestation, followed by *H. kunzi* 50.0 % (1/2). One third of *Balionycteris seimundi* (1/3), *H. dyacorum* (1/3) and *H. larvatus* (3/9) had bat fly infestation, followed by *Cynopterus horsfieldii* 28.6 % (2/7) and *Cynopterus brachyotis* 14.3 % (1/7). There was no bat fly found on *Kerivoula minuta* and *Nycteris tragata*.

The present study collected a total of 98 bat flies (n = 98) and these bat flies were grouped into two families, comprising 79.6 % (78/98) from the family Nycteribiidae (Fig. 1) and 20.4 % (20/98) from Streblidae (Fig. 2). Kelantan had three bat fly species among 71 bat flies sampled, while the 27 bat flies collected from Terengganu consisted of four different species (Table 1). *R. affinis* was the only bat species with 2–3 bat fly species co-infestations. Kelantan's most abundant bat fly species was *Eucampsipoda* cf. *sundaica*, followed by *Phthiridium fraternum* and *Brachytarsina* cf. *modesta*. While in Terengganu, *Brachytarsina* cf. *modesta* was the most abundant species, followed by *Phthiridium* spp., *Raymondia* spp. and *Leptocyclopodia* spp.

Molecular detection for *Bartonella* was performed using two separate PCR assays, one targeting the *Bartonella* 16S–23S ribosomal RNA ITS and the other targeting *Bartonella gltA*. Amplification was successful with both PCR assays, however poor sequencing signals were obtained for *gltA* PCR products, hampering identification. Among the 39 bat fly pools, 66.7 % (26/39) were found positive for *Bartonella* spp. Out of the 26 positive pools, 73.3 % (11/15) samples from Terengganu and 62.5 % (15/24) samples from Kelantan were positive for *Bartonella* spp. by the amplification of the *Bartonella* 16S–23S ribosomal RNA ITS (Table 2). From the PCR-positive pools, five pools were randomly selected (RE5, RE7, RE10, SD1 and SA1) for DNA sequencing due to funding constraints. These PCR-positive pools produced bands with product sizes of 550bp (SD1) and 380bp (RE5, RE7, RE10 and SA1) which were not the expected 408–717bp band size. Nevertheless, sequencing results of these products confirmed that they belonged to *Bartonella* spp. Results showed that bat flies from both families were potential vectors for *Bartonella* spp. as 75.9 % (22/29) and 40.0 % (4/10) pooled samples of Nycteribiidae and Streblidae were detected with *Bartonella*.

BLAST results of the sequences from the five positive samples for *Bartonella* showed that three pools (RE5, RE7 and RE10) from Gunung Reng, Kelantan, have the closest sequence identities (99.0 %) with *Bartonella* spp. Lisso-Nig-922 from Nigeria (Accession no. MN504709). The PCR-positive pools from Sekayu Terengganu (SD1 and SA1) were closely related to *Bartonella* spp. strain KP277 from Thailand (Accession no. KY232247) and *Bartonella* spp. Rhin-3 from the Republic of Georgia (Accession no. MN258141) with 99.0 % and 100 % sequence identity, respectively. Fig. 3 revealed the dendrogram constructed based on the partial *Bartonella* 16S–23S ribosomal RNA ITS sequences of RE5, RE7, RE10, SD1, SA1 and *Bartonella* reference strains from the NCBI GenBank. The results also demonstrated that these *Bartonella* sequences were very different (<70 % identities) than previously reported *Bartonella* strains that were detected in bats and insects (tick and bat flies) from Malaysia. The sequences were deposited in the GenBank with the accession numbers OR523827 – 523831. The sequence similarity between SD1 and SA1 were less than 60 %, while all of the *Bartonella* spp. from Gunung Reng, Kelantan were closely related with more than 99 % identity. Our findings indicate that there are two strains of *Bartonella* spp. circulating in Sekayu, Terengganu and only one strain in Gunung Reng, Kelantan.

**Table 1**
Bat fly species and its associated host collected from the east coast of Peninsular Malaysia.

| Locality | Bat Species (n) | Bat Fly Species |
|---|---|---|
| Gunung Reng, Kelantan | *Eonycteris spelaea (57)* | *Eucampsipoda* cf. *sundaica* |
| | *Rhinolophus affinis (32)* | *Phthiridium fraternum* |
| | | *Brachytarsina* cf. *modesta* |
| | *Rhinolophus pusillus (2)* | *Phthiridium* sp. |
| | *Rhinolophus stheno (4)* | *Brachytarsina* cf. *modesta* |
| | *Hipposideros dyacorum (2)* | No Bat Fly Found |
| | *Hipposideros kunzi (1)* | |
| | *Hipposideros larvatus (2)* | |
| | *Myositis muricola (1)* | |
| | *Taphozous melanopogon (2)* | |
| Sekayu Recreational Forest, Terengganu | *Balionycteris seimundi (3)* | *Leptocyclopodia* sp. |
| | *Cynopterus brachyotis (7)* | *Leptocyclopodia ferrarii* |
| | *Cynopterus horsfieldii (7)* | *Leptocyclopodia ferrarii* |
| | *Hipposideros dyacorum (3)* | *Raymondia* sp. |
| | *Hipposideros kunzi (2)* | *Raymondia pagodarum* |
| | *Hipposideros larvatus (9)* | *Phthiridium* cf. *euxestum* |
| | *Rhinolopus affinis (19)* | *Brachytarsina* cf. *modesta* |
| | | *Phthiridium* cf. *euxestum* |
| | | *Raymondia pseudopagodarum* |
| | *Kerivoula minuta (2)* | No Bat Fly Found |
| | *Nycteris tragata (1)* | |
| Pahang National Park, Merapoh, Pahang | *Hipposideros armiger (1)* | No Bat Fly Found |
| | *Hipposideros kunzi (12)* | |
| | *Hipposideros larvatus (16)* | |
| | *Rhinolophus stheno (1)* | |

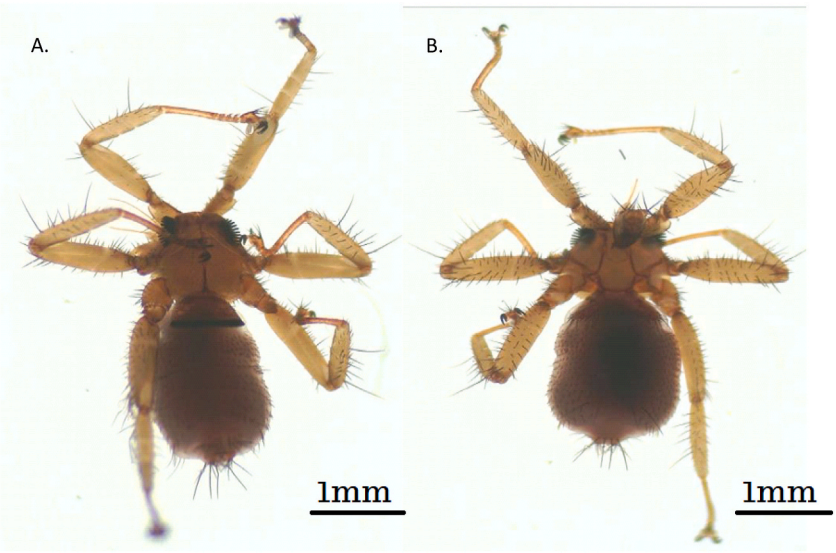


**Fig. 1.** Bat fly, *Eucampsipoda* cf. *sundaica* under the Nycteribiidae family collected from *Eonycteris spelaea* in Gunung Reng, Kelantan. A. Dorsal view (left) and B. Ventral view (right).


**Fig. 2.** Bat fly, *Raymondia* sp. under the Streblidae family collected from *Hipposideros dyacorum* in Sekayu, Terengganu. A. Dorsal view (left) and B. Ventral view (right).

## 4. Discussion

Bat flies from the families Nycteribiidae and Streblidae in Malaysia have been poorly studied, and there have been no recently published works of literature on this group from Malaysia since the late 19th century. The most recent publication on the checklist of bat flies and their associated bat hosts in Malaysia recorded 15 bat fly species from 24 species of bats based on the survey conducted at 10 localities [35]. From the three localities surveyed in this study, 15 bat species were recorded, with only 10 bat species infested with bat flies. The 10 species of bat flies found in this study somehow reflected a high species ratio of bat fly to bat species: 1.0 (10/10) in this study as compared to 0.6 (15/24) in Azhar et al. [35].

The possibility of bats acting as the asymptomatic reservoir for *Bartonella* and bat flies as the vector has been mooted previously and is currently supported by the increasing number of reported cases across several continents, including Asia such as in Taiwan [36], Vietnam [37], Thailand [38], China [39], Japan [40,41] and Malaysia [18,19]. Various studies also revealed a genetically diverse pool of *Bartonella* lineages in bats and their arthropod vectors in multiple countries [42–44]. Some of these *Bartonella* spp. are potentially zoonotic in which bats have been proven as the reservoir hosts, and bat flies as the vectors in transmitting *Bartonella* spp. to new hosts [13,45,46].

*Eonycteris spelaea* is the most abundant bat species recorded in this study, with the most infested individuals. The roosting behaviour in large single-species colonies [9] and higher degree of roost fidelity [47] might facilitate increased contact between bats leading to higher infestation rates [48]. Similar to the previous record, *Eucampsipoda sundaica* is the sole ectoparasite of the Dawn bat, *E. spelaea* [49] in Peninsular Malaysia. This bat species seems to be the common host species for *E. sundaica,* which has also been

**Table 2**
Bat fly species PCR-positive for *Bartonella* with their associated bat species and localities.

| Bat Species | Bat Fly Species (Family) | Pool Samples (n) | *Bartonella* PCR-positive (n) |
|---|---|---|---|
| **Kelantan** | | | |
| *E. spelaea* | *Eucampsipoda* cf. *sundaica* (N) | 17 | 13 |
| *R. affinis* | *Phthiridium fraternum* (N) | 3 | 2 |
| | *Brachytarsina* cf. *modesta* (S) | 2 | 0 |
| *R. pusillus* | *Phthiridium* sp. (N) | 1 | 0 |
| *R. stheno* | *Brachytarsina* cf. *modesta* (S) | 1 | 0 |
| **Terengganu** | | | |
| *B. seimundi* | *Leptocyclopodia* sp. (N) | 1 | 0 |
| *C. brachyotis* | *Leptocyclopodia ferrarii* (N) | 1 | 1 |
| *C. horsfieldii* | *Leptocyclopodia ferrarii* (N) | 2 | 2 |
| *H. dyacorum* | *Raymondia* sp. (S) | 1 | 1 |
| *H. kunzi* | *Raymondia pagodarum* (S) | 1 | 0 |
| *H. larvatus* | *Phthiridium* cf. *euxestum* (N) | 1 | 1 |
| *R. affinis* | *Phthiridium fraternum* (N) | 3 | 3 |
| | *Raymondia pseudopagodarum* (S) | 3 | 3 |
| | *Brachytarsina* cf. *modesta* (S) | 2 | 0 |
| | | 39 | 26 |

N: Nycteribiidae; S: Streblidae.

reported in Thailand, Malaysia, Singapore [48] and Indonesia [50]. *Bartonella* has been reported in both this bat and bat fly species in Indonesia and China [50,51].

*Rhinolophus affinis* was the only bat species with different bat fly species co-infestations. Previous research by Li et al. [52] reported *Bartonella* detection in 50.0 % (22/44) of *R. affinis* blood samples indicating that *R. affinis* is one of the reservoirs for *Bartonella*. Out of the three species of bat flies collected from *R. affinis* in this study, only *Brachytarsina* cf. *modesta,* collected from two different localities did not carry *Bartonella*. *Brachytarsina* cf. *modesta* were previously sampled from several hosts, namely, *Aselliscus stoliczkanus* and from another nine species of *Rhinolophus,* inferring that *B.* cf. *modesta* exhibits a polyxenous relationship [48,53]. Host specificity influences the prevalence of *Bartonella* infection in bat flies; a higher prevalence of *Bartonella* infection is usually associated with monoxenous or oligoxenous bat fly species [54]. This also explains why *Leptocyclopodia ferrarii,* which is relatively host-specific to *Cynopterus* bats, were all positive for *Bartonella* [19,35,55].

The molecular prevalence of *Bartonella* in bat flies from this study was 66.7 % (26/39 pools). This result was higher than the previous study conducted by Low et al. [19] in which 31.1 % of *L. ferrarii* (14/45) were detected with *Bartonella*. The observed difference in *Bartonella* prevalence may be attributed to factors such as the sampling site. The prior study focused on an oil palm plantation, examining only one bat species and one bat fly species. Ecological variations in the forest reserve, a natural habitat, where this study was conducted, might contribute to the higher prevalence of *Bartonella* compared to the oil palm plantation [56]. The use of sample pooling methods in this study may also be a contributing factor to the higher *Bartonella* prevalence. For logistical and economic reasons, pool screening is a common method for analyzing arthropod vectors in veterinary and human medicine [57]. However, as the number of positive samples increases, there is a growing risk of misestimating the number of positive samples [58].

*Bartonella* is a highly diverse bacteria genus, and bats have been identified as possible reservoirs for novel *Bartonella* species. Sequence and phylogenetic analyses of the *Bartonella* spp. amplified in the present study suggest that genetic diversity is substantial since the *Bartonella* sequences were similar to strains from Africa, Europe and Southeast Asia [38,59]. This finding corroborates the results by Low et al. [19] indicating that *Bartonella* in bats and bat flies in Southeast Asia is highly diverse and requires more studies. Despite being host-specific, *Bartonella* carried by bats can potentially spill over into the human population. The case of human infection with a novel *Bartonella mayotimonensis* in the USA that was later isolated in bats in Europe [13] is a case in point. This case underscores the importance of researching the presence of *Bartonella* spp. in the local bat population to establish baseline information for *Bartonella* carried by wildlife in Malaysia.

## 5. Conclusion

This study highlights the poorly studied bat fly population in Malaysia and their potential for transmitting zoonotic diseases, particularly those related to the *Bartonella* spp. The data collected in this study indicate a high prevalence of bat flies infesting specific bat species, with some bats showing co-infestations by different bat fly species. The genetic diversity of *Bartonella* detected in this study underscores the importance of studying and monitoring these bacteria in the local bat populations. The potential for *Bartonella* spp. to spill over into the human population, emphasizes the significance of establishing baseline information regarding *Bartonella* carried by wildlife in Malaysia.

## Data availability statement

Data included in article/supp. material/referenced in article.

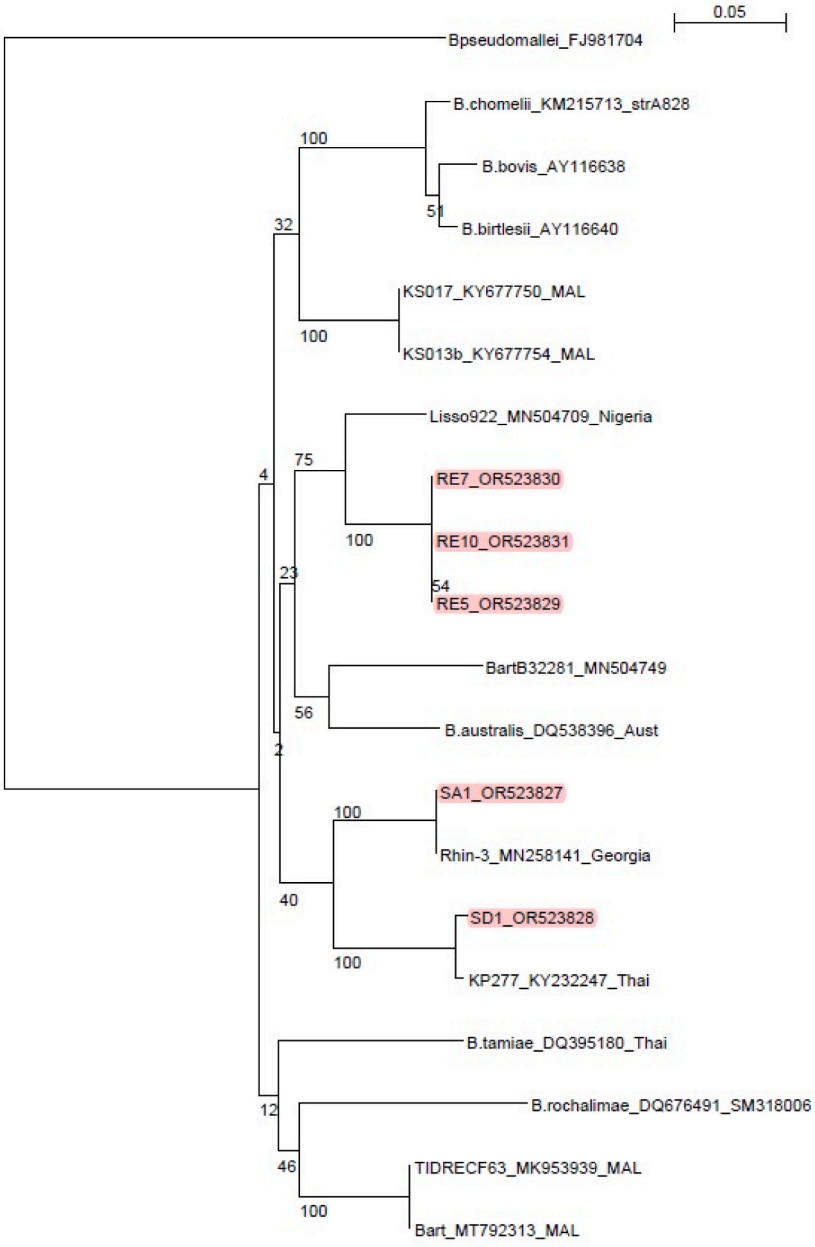


**Fig. 3.** Phylogenetic tree of *Bartonella* spp. from this study (RE5, RE7, RE10, SD1 and SA1 were highlighted in red) with *Bartonella* reference strains (with their accession numbers) from NCBI Genbank based on partial *Bartonella* 16S–23S ribosomal RNA ITS sequences. *Burkholderia pseudomallei* from Malaysia (Accession no. FJ981704) was used as an outgroup. Scale bar (0.05) indicates nucleotide substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

## CRediT authorship contribution statement

**Tan Li Peng:** Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization. **Azra Hafizah Kamar:** Writing – original draft, Investigation. **Maizan Mohamed:** Writing – review & editing, Funding acquisition. **Brenda Gilbert:** Resources, Investigation. **Nani Izreen Mohd Sani:** Investigation. **C.W. Salma C.W. Zalati:** Investigation. **Ruhil Hayati Hamdan:** Writing – review & editing. **Abdulloh Samoh:** Investigation. **Shih Keng Loong:** Writing – review & editing, Investigation, Funding acquisition.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Maizan Mohamed reports equipment, drugs, or supplies and travel were provided by Malaysia Ministry of Higher Education. Shih Keng Loong reports equipment, drugs, or supplies and travel were provided by Malaysia Ministry of Higher Education. Maizan Mohamed, Shih Keng Loong reports a relationship with Malaysia Ministry of Higher Education that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29785.

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