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Relationship among bats, parasitic bat flies, and associated pathogens in Korea

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Abstract

Background: Bats are hosts for many ectoparasites and act as reservoirs for several infectious agents, some of which exhibit zoonotic potential. Here, species of bats and bat flies were identified and screened for microorganisms that could be mediated by bat flies.

Methods: Bat species were identified on the basis of their morphological characteristics. Bat flies associated with bat species were initially morphologically identified and further identified at the genus level by analyzing the cytochrome c oxidase subunit I gene. Different vector-borne pathogens and endosymbionts were screened using PCR to assess all possible relationships among bats, parasitic bat flies, and their associated organisms.

Results: Seventy-four bat flies were collected from 198 bats; 66 of these belonged to Nycteribiidae and eight to Streblidae families. All Streblidae bat flies were hosted by Rhinolophus ferrumequinum, known as the most common Korean bat. Among the 74 tested bat flies, PCR and nucleotide sequencing data showed that 35 (47.3%) and 20 (27.0%) carried Wolbachia and Bartonella bacteria, respectively, whereas tests for Anaplasma, Borrelia, Hepatozoon, Babesia, Theileria, and Coxiella were negative. Phylogenetic analysis revealed that Wolbachia endosymbionts belonged to two different superfamilies, A and F. One sequence of Bartonella was identical to that of Bartonella isolated from Taiwanese bats.

Conclusions: The vectorial role of bat flies should be checked by testing the same pathogen and bacterial organisms by collecting blood from host bats. This study is of great interest in the fields of disease ecology and public health owing to the bats’ potential to transmit pathogens to humans and/or livestock.

Keywords: Bat, Bat fly, Blood-borne pathogen, Phylogeny, Prevalence

Background

As a group, bats include approximately 1432 species [1]. Several bat species are key to their ecosystems and also act as pathogen reservoirs [2]. Bat viruses are of great interest in disease ecology and public health owing to their potential to infect humans and livestock [3]. Moreover, bacteria and protozoa have also been detected in bats. Bartonella bacteria and Trypanosoma cruzi protozoa, which are associated with bats, have also been detected in humans, making these bat-related organisms an urgent public health concern [4, 5]. Bats harbor several ectoparasites, including bat flies, fleas, and certain arachnids, such as mites and ticks. Bat fly families Nycteribiidae and Streblidae belong to the superfamilies Hippoboscoidea, which also includes the families Hippoboscidae (louse flies and keds) and Glossinidae (tsetse flies), and are the most common bat ectoparasites [2, 6].

Currently, 275 species of 21 genera of nycteribiids and 227 species of 31 genera of streblids have been described [7]. The importance of louse flies as a potential vector has been well studied. Recently, it has been confirmed...
that *Anaplasma ovis*, *Bartonella* spp., *Rickettsia* spp., and *Trypanosoma* spp. are present in these insects [8–10]. Bat flies are also considered vectors. In recent studies, bat ectoparasite burden was found to be proportional to *Bartonella* infection; moreover, *Bartonella* spp. were also detected in bat flies and host bats, underscoring the parasite vector potential. However, more research is warranted [11, 12]. Furthermore, it has not been demonstrated that bat flies transmit *Bartonella* bacteria. The vector potential of bat flies was demonstrated only in *Polychromophilus* spp. [13].

Bat flies are obligate ectoparasites for bats and include endosymbiotic prokaryotes that are not yet well understood; however, it is assumed that they establish a symbiotic relationship with mutualistic bacteria [14]. Members of the superfamily Hippoboscoidea require milk secretion for larval development, and certain bacteria such as *Bartonella* and *Wolbachia* can be vertically transmitted through this process. These bacteria can also be horizontally transmitted through parasitoids or via contact with contaminated saliva [15, 16]. However, horizontal transmission has not been proven in bat flies or any other hippoboscids.

*Wolbachia* is a bacterium belonging to the order Rickettsiales, which includes the genera *Anaplasma*, *Ehrlichia*, and *Rickettsia*. *Wolbachia* influences host reproduction through extensive symbiotic interactions in some species and is estimated to be present in up to 66% of insect species [17]. *Wolbachia* has become an integral component of vector-mediated disease control due to its ability to spread through insect populations and influence vector competence through pathogen protection [18]. *Bartonella* spp. are parasitic bacteria that infect the red blood cells of vertebrates. Several different bacterial species [19], including *Bartonella mayotimonensis*, are associated with bats, some of which are zoonotic and can cause disease in humans [5].

However, to date, few studies have examined the pathogenic relationships among bat flies, although previous studies reported the possibility of *Bartonella* and *Wolbachia* bacteria occurring transiently [20, 21]. In general, the high degree of host specificity in bat flies reduces the likelihood of interspecies transmission of pathogens, but bat flies are still likely to carry transmissible pathogens within the host population [7]. Most previous studies on microorganisms, including those on *Wolbachia* spp., in bat flies have focused on endosymbiotic characteristics or distribution [2, 20].

In Korea, many vector-mediated diseases and causative agents, including *Anaplasma*, *Bartonella*, and *Borrelia*, occur in humans [22–24]. However, there are few data regarding bats and bat flies in Korea. Furthermore, a recent Korea-focused study assessed the local distribution of bat flies without considering the pathogens mediated by these flies [25]. Therefore, the purpose of this study was to investigate the relationships among bats, parasitic bat flies, and their associated bacteria in Korea.

**Methods**

**Study area, sample collection, and species identification of bats and bat flies**

Bats and bat flies were collected from caves, forests, and abandoned mines in 12 cities across seven provinces [Gangwon (Inje), Chungbuk (Danyang), Gyeongbuk (Yeongju, Andong, Yeongcheon, and Gyeongju), Ulsan, Jeonbuk (Sunchang), Gwangju, and Jeonnam (Muan, Jindo, and Wando)] of Korea from February 2016 to August 2017 (Fig. 1). A total of 198 dead bats were found, and 74 bat flies were collected and immersed in 70% ethyl alcohol solution. The bat flies were collected by ecologists licensed from the National Institute of Environmental Research, Korea. The bat species were identified based on their morphological characteristics as previously described [26, 27], and all collection-related information was provided from the ecologists.

Bat fly species were initially identified using key morphological characteristics, such as the presence or absence of wings, using a dissecting microscope (Fig. 2) [28]. The species were further identified at least at the genus level by analyzing the cytochrome c oxidase subunit I (COI) gene (approximately 658 bp length) [29, 30], which was amplified through PCR using invertebrate-specific primers [31, 32].

**DNA extraction and PCR assay**

Bat fly DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) as per the manufacturer’s instructions. An Infinite® 200 PRO NanoQuant (Tecan, Männedorf, Switzerland) plate reader was used to assess the quality and quantity of bat fly DNA by calculating the ratio of the absorbance at 260 nm and 280 nm. DNA samples were stored at −20 °C until further use.

The commercially available AccuPower® HotStart PCR Premix kit (Bioneer, Daejon, Korea) was used for PCR. This premix product includes most of the elements required for PCR, including DNA polymerase, dNTPs, reaction buffer, and metal ions, lyophilized in a single tube. For PCR amplification, primers, DNA template, and distilled water are added until the total volume of the mixture reaches 20 μl. Bat fly-mediated pathogens and bacterial organisms were detected by amplification using primers specific to a target gene in each microorganism. All reactions were performed using 20 μl reaction mixture containing 16 μl distilled water, 1 μl of 10 μM of each primer pair, and 2 μl template DNA.
We amplified the 16S rRNA regions of Rickettsiales (Anaplasma, Ehrlichia, Rickettsia, and Wolbachia species) and Coxella spp.; 5S–23S rRNA regions of Borrelia spp.; 18S rRNA regions of Babesia, Theileria, and Hepatozoon species; and internal transcribed spacer I (ITS-1) regions of Bartonella spp. [23, 33–36]. Positive DNA samples were confirmed using a second set of PCR primers to amplify other regions of the gene, including citrate synthase gene (gltA) of Bartonella spp. [37] and cell division protein FtsZ (ftsZ) of Wolbachia spp. [36]. These primers are listed in Additional file 1: Table S1, along with their expected amplicon sizes. All PCR amplifications were performed using the Mastercycler® nexus GSX1 (Eppendorf, Hamburg, Germany) under conditions outlined in Additional file 2: Table S2. The PCR products were electrophoresed on a 1% agarose gel stained with ethidium bromide. All amplicons were visualized and photographed using a UV transilluminator, and PCR-positive products were sent.
to Macrogen (Daejeon, Korea) for DNA sequencing analysis.

Sequencing and phylogenetic analysis
The obtained sequence data were aligned and edited using BioEdit 7.2.5 [38]. MEGA 7 was used to construct phylogenetic trees for each species using the maximum likelihood method with 1000 replicates based on the fragments of COI, ftsZ, and gltA of bat flies, Wolbachia spp., and Bartonella spp., respectively [39]. Reference sequence data were obtained using NCBI Web BLAST (http://www.ncbi.nlm.nih.gov/blast). The phylogenetic tree of Wolbachia spp. based on ftsZ was constructed using 27 GenBank database entries and Ehrlichia sp. as the outgroup. The phylogenetic tree of the Bartonella spp. based on gltA was constructed using 15 GenBank database entries and Brucella sp. as the outgroup.

Results
Identification of bat species
Overall, 11 species of seven genera belonging to three families were morphologically identified from 198 bats. One species each of Miniopteridae and Rhinolophidae and nine species of Vespertilionidae were found. The most common bat species was Miniopterus fuliginosus (32.8%, n = 65), followed by Rhinolophus ferrumequinum (29.3%, n = 58), Myotis macrodactylus (14.1%, n = 28), Vespertilio sinensis (7.1%, n = 14), Myotis petax (4.0%, n = 8), Eptesicus serotinus (3.5%, n = 7), Myotis bombinus (3.0%, n = 6), Murina hilgendorfi (2.5%, n = 5), Hypsugo alaschanicus (1.5%, n = 3), Myotis ikonnikovi (1.0%, n = 2), Myotis aurascens (0.5%, n = 1), and one unidentified specimen (Table 1).

Identification of bat fly species
A total of 74 bat flies were collected from 198 bats. Ectoparasites other than bat flies were not detected. Organisms belonging to the families Nycteribiidae (89.2%, n = 66) and Streblidae (10.8%, n = 8) were identified in three species of host bat (Table 2). Among the Nycteribiidae specimens, five species from three genera were identified, and the % similarities of the bat fly species based on their GenBank match were as follows: Nycteribia allotopa (100% with LC522000), Nycteribia parvula (96.7% with KF021501), Nycteribia pleuralis (94.3% with AB632553), Penicillidia jenynsii (98.6% with AB632562), and Phthiridium hindlei (99.9% with AB632569). Among the Streblidae specimens, only one species of the genus Brachytarsina was identified, and % similarity of the bat fly species based on GenBank match was Brachytarsina kanoi (93.0% with AB632571) (Fig. 3; Table 2). Although it is the closest homology to this species, there is the probability of another closely related species apart from this. Most bat fly specimens were found on M. fuliginosus (51.4%, n = 38). All P. hindlei (n = 15) and Brachytarsina sp. (n = 8) were parasitic on the bat species R. ferrumequinum. Most P. jenynsi (family Nycteribiidae) flies were collected from the specimens obtained from the Jeonbuk Province (24/26), whereas only two such individuals were identified in specimens from the Chungbuk Province. Brachytarsina sp. (family Streblidae) was identified in three specimens from

| Table 1 | Bat distribution by location |
|-----------------|-----------------------------|
| Bat species (n) | Chungbuk | Gangwon | Gwangju | Gyeongbuk | Jeonnam | Jeonbuk | Ulsan | Unknown | % |
|-----------------|---------|---------|---------|-----------|---------|---------|-------|---------|---|
| Miniopteridae (65) |        |         |         |           |         |         |       |         |   |
| Miniopterus      |         |         |         |           |         |         |       |         |   |
| Miniopterus fuliginosus (65) | 16 | 7 | 1 | 39 | 2 | 32.8 |
| Rhinolophidae (58) |        |         |         |           |         |         |       |         |   |
| Rhinolophus      |         |         |         |           |         |         |       |         |   |
| Rhinolophus ferrumequinum (58) | 4 | 4 | 6 | 8 | 9 | 12 | 15 | 29.3 |
| Vespertilionidae (74) |        |         |         |           |         |         |       |         |   |
| Eptesicus        |         |         |         |           |         |         |       |         |   |
| Eptesicus serotinus (7) | 3 | 4 |         |           |         |       | 3.5  |         |   |
| Hypsugo          |         |         |         |           |         |         |       |         |   |
| Hypsugo alaschanicus (3) | 2 |         | 1 |         |         |       | 1.5  |         |   |
| Murina           |         |         |         |           |         |         |       |         |   |
| Murina hilgendorfi (5) |         |         |         |         | 5 | 2.5  |       |         |   |
| Myotis           |         |         |         |           |         |         |       |         |   |
| Myotis aurascens (1) |         |         |         |           | 1 | 0.5  |       |         |   |
| Myotis bombinus (6) |         |         |         |           | 6 |       | 3.0  |         |   |
| Myotis ikonnikovi (2) |         |         |         |           | 2 | 1.0  |       |         |   |
| Myotis macrodactylus (28) | 3 |         | 2 | 20 | 3 | 14.1 |       |         |   |
| Myotis petax (8) |         |         |         |           | 2 |       | 4.0  |         |   |
| Vespertilio      |         |         |         |           |         |         |       |         |   |
| Vespertilio sinensis (14) | 10 |         | 4 |       | 1 | 0.5  |       |         |   |
| Unidentified (1) |         |         |         |           | 1 | 0.5  |       |         |   |
| Total            |        |         |         |           | 42 | 2 | 7 | 26 | 11 | 68 | 12 | 30 | 100 |   |
Table 2  Collected host bat and bat fly species identification

| Host bat species | No. of collected bat fly (%) | Total |
|------------------|------------------------------|-------|
|                  | Nycteribiidae (66, 89.2%)   |       |
|                  | Nycteribia allotopa          |       |
|                  | Nycteribia parvula           |       |
|                  | Nycteribia sp.               |       |
|                  | Penicillidia jenynsi         |       |
|                  | Phthiridium hindlei          |       |
|                  | Unidentified                 |       |
|                  | Brachytarsina sp.            |       |
| Nycteribiidae    |                             |       |
| Miniopterus fuliginosus | 2 (2.7) | 22 (29.7) |
|                   | 0 (0.0) | 3 (4.1) |
|                   | 0 (0.0) | 2 (2.7) |
| Myotis macrodactylus | 0 (0.0) | 2 (2.7) |
| Rhinolophus ferrumequinum | 0 (0.0) | 2 (2.7) |
| Total            | 2 (2.7) | 6 (8.1) |

* The closest GenBank matching species is *Nycteribia pleuralis*
* The closest GenBank matching species is not identified
* The closest GenBank matching species is *Brachytarsina kanoi*

Fig. 3  Phylogenetic tree based on bat fly cytochrome c oxidase subunit I gene-amplifying sequences. The ked and tsetse fly sequences were used as outgroups. Scale bar indicates an evolutionary distance of 0.20 nucleotides per position in the sequence. The black circles (●) indicate the bat fly sequences identified in this study.
Gyeongbuk, three specimens from Jeonnam, and two specimens from Ulsan (Table 3).

Screening for pathogens and endosymbionts mediated by bat flies
Of the identified pathogens and endosymbionts, 35 specimens were part of the *Wolbachia* spp. (47.3%) and 20 specimens of the *Bartonella* spp. (27.0%); no other microorganisms (such as *Coxiella*, *Borrelia*, *Anaplasma*, *Ehrlichia*, *Rickettsia*, *Hepatozoon*, *Babesia*, and *Theileria* species) were detected. Most *Wolbachia* spp. were detected in *P. jenynsii* (22/35, 64.7%), and *Bartonella* spp. were most frequently found in *P. hindlei* (10/20, 50.0%) (Table 4).

Sequence and phylogenetic analyses of bat fly-mediated pathogens and endosymbionts
*Wolbachia* spp. in *P. hindlei* share 97.7–99.8% sequence identity with *Wolbachia*-hosting spiders, moths, and beetles (GenBank: KT319069, FM883705, KC578235). *Wolbachia* spp. in *N. parvula* and *P. jenynsii* share 95.4–98.5% sequence identity with *Wolbachia* spp. isolated from cockroach and termite (GenBank: AJ292345, FJ390318, DQ457402) and 95.0–96.7% sequence identity with that isolated from nematode (GenBank: FR827926, AJ628414). Through phylogenetic analysis of the *Wolbachia* spp., the three sequences from *Phthiridium* spp. bat flies were clustered as supergroup A, and the other two sequences from *Nycteribia* spp. and *Penicillidia* spp. bat flies were clustered as supergroup F (Fig. 4) [17].

In the phylogenetic analysis of *Bartonella gltA* amplified from the bat flies in this study, six representative sequences were obtained from five bat fly species with 86.6–91.9% sequence identity (Fig. 5). *Bartonella* sequences from the bat fly *P. hindlei* (GenBank: MT362935) presented 91.9% identity with *P. jenynsii* (GenBank: MT362933), 91.7% with *N. allotopa* (GenBank: MT362931), and 86.6% with *Nycteribia* sp. (GenBank: MT362934). Particularly, one *Bartonella* sequence detected from the Japanese bat fly (GenBank: LC522030) had 100% sequence identities with the *Bartonella* sequences from this study (GenBank: MT362932 and MT362933). In addition, *Bartonella* sequences from the Taiwan bat (GenBank: JF500511), Japanese bat (GenBank: LC483820), and bat fly (GenBank: LC522032) had 100% sequence identity with a *Bartonella* sequence detected in this study (GenBank: MT362931).

Discussion
According to the morphological characteristics of bats identified in Korea, 24 species have been reported from 11 genera [26, 27]. According to the key to the order Chiroptera in Korea, four bat families (Miniopteridae, Molossidae, Rhinolophidae, and Vespertilionidae) have been reported in Korea. In this study, 11 species

| Bat fly species | Bat host species (n) | Chungbuk | Gyeongbuk | Jeonbuk | Jeonnam | Ulsan | Unknown |
|----------------|---------------------|----------|----------|--------|--------|------|---------|
| Nycteridae (66) |                     |          |          |        |        |      |         |
| *Nycteribia* albotapa (2) | *Miniopterus fuliginosus* (2) | 1 | 1 |        |        |      |         |
| *Nycteribia* parvula (2) | *Miniopterus fuliginosus* (2) | 1 | 1 |        |        |      |         |
| *Nycteribia* sp. (6) | *Miniopterus fuliginosus* (2) | 1 | 1 |        |        |      |         |
| *Nycteribia* (2) | *Miniopterus fuliginosus* (2) | 2 |      |        |        |      |         |
| *Penicillidia jenynsii* (26) | *Miniopterus fuliginosus* (22) | 20 | 20 |        |        |      |         |
| *Penicillidia* (3) | *Miniopterus fuliginosus* (3) | 3 |        |        |        |      |         |
| *Penicillidia* (1) | *Miniopterus fuliginosus* (15) | 1 |        |        |        |      |         |
| *Phthiridium* hindlei (15) | *Rhinolophus ferrumequinum* (15) | 3 | 3 | 3 | 5 | 1 |         |
| Unidentified (3) | *Rhinolophus ferrumequinum* (5) | 3 | 2 |        |        |      |         |
| Streblidae (8) | *Rhinolophus ferrumequinum* (8) | 5 | 3 |        |        |      |         |
| *Brachytarsina* sp. (8) | *Rhinolophus ferrumequinum* (8) | 3 | 3 |        |        |      |         |
| Total (74) |                     | 14 | 6 | 38 | 8 | 7 | 1 |

* Not detected in Gangwon and Gwangju regions
* The closest GenBank matching species is *Nycteribia pleuralis*
* The closest GenBank matching species is not identified
* The closest GenBank matching species is *Brachytarsina kanoi*
belonging to seven genera in three families (Miniopteri-
dae, Rhinolophidae, and Vespertilionidae) were identi-
fied. The collection places of bats were recorded for all
except 31 specimens (these specimen data on tubes were
erased because of leakage of ethanol during transfer),
most of which were found in mines (79 specimens), caves
(76 specimens), and the rest forests (12 specimens). Rhi-
nolophus ferrumequinum was found in all regions except
Gangwon; however, this does not necessarily mean that
it does not occur in Gangwon. Only two bat specimens
were collected from Gangwon. Rhinolophus ferrumequi-
um is known to be the most widely occurring bat in
Korea [26].

A total of 74 bat flies were collected. One possible rea-
son for this small number could be that the flies were col-
lected from dead bats. Therefore, several bat flies would
have left after the host bat died.

Molecular identification and phylogenies of bat flies
have been widely utilized over the last decade to char-
acterize different fly species [40, 41]. COI, in particular,
has been proven a useful marker in the documentation
of invertebrates and insects [41–43]. Previous studies
on Korean bat fly were limited to morphological char-
acteristics [25]. Moreover, traditional morphological
identification methods are extremely time-consuming
because bat flies are complex, extremely small, and
diverse in species. Although this does not justify the lack
of morphology-based identification, our results showed
that a molecular approach would be useful in the quick
identification of the different species of bat flies, at least
at the genus level. There was a limitation that COI was
not sufficient to reliably identify bat flies by itself at the
species level because sequence data of many species are
still missing from the GenBank. Therefore, in this study,
COI sequencing enabled genus-level identification and
indicated the closest GenBank match species. In some
species, the most similar GenBank sequences showed a
similarity of <95% (N. pleuralis, 94.3%; B. kanoi, 93.0%),
whereas others showed a similarity of >98% (N. allotopa,
100%; P. jenynsii, 98.6%; P. hindlei, 99.9%). Therefore,
more comparable data on COI will allow a clear distinc-
tion of bat flies at the species level. Previously, COI was
also used for species identification in other families of
Diptera [44, 45].

Phthiridium hindlei is an ectoparasite of R. ferrumequi-
um, which has not been reported in Korea previously.
Most Nycteribia spp. were detected in M. fuliginosus
(8/12, 66.7%), which is consistent with the findings of a
previous study that collected Nycteribia spp. from Mini-
opterus sp. in Korea [25].

Penicillidia jenynsii (family Nycteribiidae) was mostly
collected from the Jeonbuk Province (24/26), with only
two individuals identified in the Chungbuk Province.
Brachytarsina sp. (family Streblidae) was confirmed only

| Bat fly species | Bat host species (n) | Wolbachia | Bartonella |
|----------------|---------------------|-----------|-----------|
| Nycteribiidae  |                     |           |           |
| Nycteribia allotopa (2) | Miniopterus fuliginosus (2) | 0 | 1 |
| Nycteribia parvula (2) | Miniopterus fuliginosus (2) | 2 | 0 |
| Nycteribia sp. a (6) | Miniopterus fuliginosus (2) | 0 | 1 |
| Nycteribia sp. b (2) | Myotis macrodactylus (3) | 0 | 1 |
| Penicillidia jenynsii (26) | Rhinolophus ferrumequinum (1) | 0 | 0 |
| Phthiridium hindlei (15) | Rhinolophus ferrumequinum (1) | 10 | 10 |
| Unidentified (13) | Rhinolophus ferrumequinum (5) | 1 | 1 |
| Subtotal (66) | Miniopterus fuliginosus (8) | 0 | 1 |
| Total (74) |                        | 35 (53.0) | 18 (27.3) |

| Streblidae |                     |           |           |
| Brachytarsina sp. c (8) | Rhinolophus ferrumequinum (8) | 0 | 1 |
| Subtotal (8) |                        | 0 (0.0)  | 2 (25.0)  |
| Total (74) |                        | 35 (47.3) | 20 (27.0) |

*The closest GenBank matching species is Nycteribia pleuralis
^The closest GenBank matching species is not identified
The closest GenBank matching species is Brachytarsina kanoi
in Jeonnam, Gyeongbuk, and Ulsan Provinces, but not in Jeonbuk and Chungbuk Provinces. However, because there is a history of discovery of Brachytarsina sp. in Jeju Island and Gangwon Province [25] in the southernmost and northernmost regions of Korea, respectively, more population studies on ectoparasites and identification are required.

This study confirmed the high prevalence of Wolbachia and Bartonella bacteria and that P. hindlei was highly coinfected with Wolbachia and Bartonella spp. (9/10). According to the identification results of these pathogens and endosymbionts in this study, Wolbachia spp. was identified at a rate of 53.0% (35/66) in the family Nycteribiidae [Penicillidia sp. (22/26, 84.6%), Nycteribia spp. (2/12, 16.7%), Phthiridium sp. (10/15, 66.7%) and an unidentified Nycteribiidae species (1/13, 7.7%)] but not in the family Streblidae (Brachytarsina sp.). This is believed to be associated with the vertical transfer of the endosymbiont from mother to offspring through the mammary glands. Endosymbiont localization is consistently observed in all nycteribiid bat flies. In particular, P. jenynsis females exhibit a different pattern from that of males. In the abdominal cavity of females, larvae were found around the mammary glands, which supplied secretions and exhibited endosymbiont signals [20]. Bartonella spp. were identified at a rate of 27.3% (18/66) from the family Nycteribiidae [Phthiridium sp. (10/15, 60.7%), Nycteribia spp. (3/12, 25.0%), Penicillidia sp. (3/26, 11.5%), and an unidentified Nycteribiidae species (2/13, 15.4%)] and at a rate of 25.0% (2/8) in the family Streblidae (Brachytarsina sp.).

A previous study reported that the most common microparasites in bat flies were bacteria (n = 149), with high numbers of Bartonella spp. (n = 91, 61.0%) but few Wolbachia spp. (n = 8, 5.4%) [2]. However, in this study, Wolbachia spp. were detected at a higher rate (35/74, 47.3%), whereas Bartonella bacteria detection was less frequent (20/74, 27.0%). This could be due to the differences in sample collection time and areas.

As per the phylogeny results of Wolbachia and Bartonella bacteria, we confirmed that each species of bat flies clustered as separated roots of each type. The Wolbachia endosymbionts detected in our study were clustered into two supergroups, A and F. Supergroup A was found in arthropods, whereas supergroup F was found in both filariae and arthropods [17]. All supergroup A...
endosymbionts were detected in *P. hindlei*. Supergroup F endosymbionts were detected in other bat flies (*Penicillidia* sp. and *Nycteribia* spp.). The presence of *Wolbachia* spp. confirmed that various arthropods could be vectors, and the bat fly *Wolbachia* spp. could have sequences similar to both filariae and arthropods. In addition, the possibility of *Wolbachia* bacteria transmission through bat blood should be studied to determine the connection between bat flies and bats.

*Bartonella* endosymbionts were also grouped according to their bat fly host except *P. hindlei*. However, these sequences clustered into distinct *Bartonella* groups and were considered a separate *Bartonella* species because of its < 96% identity [46, 47]. Interestingly, in this study, with two separate branch groups, *Bartonella* host specificity in bats and bat flies was confirmed; one is the *P. jenynsii* group and the other *N. allotopa* group, all of which had *Mini-optimus* sp. bat hosts. In a previous study conducted in northern China, bat and *Bartonella* host specificity was recorded [48], and it was suggested that *N. allotopa* is the vector for transmitting *Bartonella* in bats [49]. However, the correlation between *Bartonella* species originating from bats and other bat fly species (another *Nycteribia* sp. and *P. hindlei*) requires further study.

*Bartonella* bacterial infection has been reported in humans and animals in African countries, where it was observed that a large number of local bat flies were positive for *Bartonella* spp. [50]. Bat ectoparasites generally exhibit high host specificity; therefore, their impact on other animal species and humans may be low, but the spread of bat-borne *Bartonella* spp. poses a global risk [29, 51]. Furthermore, it must be considered that endosymbionts of bat flies may come from their bat hosts. Recent research suggests that bat flies transfer viruses to host bats as well as humans [7]. *Nycteribiids* are known to host several *Bartonella* spp., and bat and *Bartonella* bacteria associations have been studied in several parts of the world, including Asia [30]. In this study, our data indicated that *Wolbachia* and *Bartonella* bacteria are associated with bat fly species. In

### Table 1: Microorganism and Host Association

| Country       | Host                                                                 |
|---------------|----------------------------------------------------------------------|
| Japan         | bat fly (*Penicillidia jenynsii*) from bat (*Miniopterus schreibersii*) |
| Japan         | bat fly (*Penicillidia jenynsii*) from bat (*Miniopterus fuliginosus*) |
| Madagascar    | bat fly (*Nycteribia stylidoptera*)                                  |
| China         | bat (*Myotis fimbrisus*)                                            |
| Japan         | bat fly (*Nycteribia allotopa*) from bat (*Miniopterus fuliginosus*)  |
| Taiwan        | bat (*Miniopterus schreibersii*)                                    |
| Japan         | bat fly (*Nycteribia altalopha*) from bat (*Miniopterus schreibersii*) |
| China         | bat (*Rhinolophus puillii*)                                         |
| China         | bat fly (*Phthirium hindlei*) from bat (*Rhinolophus ferrumequinum*) |
| Vietnam       | bat (*Rhinolophus acuminaurus*)                                     |
| Peru          | human (*Homo sapiens*)                                              |
| France        | cat                                                                  |
| Costa Rica    | bat (*Artibeus lubricus*)                                            |
| New Caledonia | cattle                                                               |
| Benin         | cattle                                                               |
| Israel        | gerbil (*Gerbillus asleandsoni*)                                     |
| USA           | human (*Homo sapiens*)                                              |
| USA           | rat (*Rattus norvegicus*)                                           |
| Korea         | mouse (*Aporobus agrarius*)                                          |
| Brazil        | bat fly (*Streblea guajino*) from bat (*Carollia perspiculus*)      |
| Israel        | fleas (*Symostompuus oleopastrei*)                                  |
| Brazil        | rodent                                                               |
| China         | bat (*Myotis paquinu*)                                              |
| China         | human (*Homo sapiens*)                                              |
| China         | bat (*Myotis fimbriatus*)                                            |

![Fig. 5](image_url) A phylogenetic tree was constructed with the *Bartonella* gltA gene-amplifying sequences generated in this study using the maximum likelihood method based on the Tamura-Nei model (1000 replicates). Sequences identified in this study are indicated by black circles (●) with isolated ID and host species scientific name. The *Brucella* sequences were used as outgroup.
this regard, studies on bat fly-mediated pathogens and endosymbionts are of great public health significance and require continued interest and research.

**Conclusions**

This study employed morphological and molecular techniques to identify bat fly species in Korea. We also determined the distribution of *P. hindlei* and its endosymbionts. Using molecular methods, we identified several microorganisms, such as the endosymbiotic *Wolbachia* and possibly pathogenic or endosymbiotic *Bartonella* of bat flies, that are parasites for Korean bats. This is the first study to use such methods to identify Korean bat flies. Although the possibility of pathogen transmission through direct contact with a bat fly is low, subsequent studies on bat blood are required to confirm the potential for direct infection between bats and bat flies. This is an important public health concern owing to its potential for transmission to other species through bats.

**Abbreviations**

COI: Cytochrome c oxidase subunit I, ITS-1: Internal transcribed spacer I.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s13071-021-05016-6.

**Additional file 1: Table S1.** Primers used for species identification of bat flies and pathogen detection in bat flies.

**Additional file 2: Table S2.** PCR conditions.

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Not applicable.

**Authors’ contributions**

All authors have seen and approved the manuscript. HL and DK did experimental concept design and wrote the manuscript. HL, MGS, SHL, JKO, SHK, WHJ, ODK, and DK provided experimental data analysis and reviewed the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

Data supporting the conclusions of this article are included within the article. The newly generated sequences were submitted to the GenBank database under the accession numbers: MT362937–MT362950 (COI), MT362926–MT362930 (ftsZ), and MT362931–MT362936 (gltA). The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

**Declarations**

**Ethics approval and consent to participate**

The bat flies used in this study were collected under the supervision of the National Institute of Environmental Research and the Ministry of the Environment in Korea. The collected bat flies were attached to the bodies of dead bats in caves, forests, and abandoned mines. Bat fly collection was performed by ecologists as permitted by the Ministry of Environment in Korea for the purpose of disease monitoring.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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