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RESEARCH

# Molecular identification of fruit bats, natural host of Nipah virus in Bangladesh, based on DNA barcode

Md. Maharub Hossain Fahim[1†], Walid Hassan[1], Afia Afsin[1], Md. Mahfuzur Rahman[2], Md. Tanvir Rahman[3], Sang Jin Lim[4*], Yeonsu Oh[5], Yung Chul Park[4], Hossain Md. Faruquee[1†] and Md. Mafizur Rahman[1*]

[1]Department of Biotechnology and Genetic Engineering, Faculty of Biological Science, Islamic University, Kushtia 7100, Bangladesh
[2]International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), Dhaka 1212, Bangladesh
[3]Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
[4]College of Forest & Environmental Sciences, Kangwon National University, Chuncheon 24341, Republic of Korea
[5]College of Veterinary Medicine & Institute of Veterinary Science, Kangwon National University, Chuncheon 24341, Republic of Korea

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***Corresponding authors**
Md. Mafizur Rahman
**E-mail** mmrahman@btge.iu.ac.bd
Sang Jin Lim
**E-mail** sangjin@kangwon.ac.kr

†These authors contributed equally to this work.

**Background:** Fruit bats are natural carriers of Nipah virus (NiV). The primary objective of this study is to identify potential reservoir species in a selected geographic regions. It is necessary to determine an accurate species identification of the associated reservoir bat species distributed in a specific region.
**Results:** In this study, we collected 20 different bat specimens from the NiV-prone area of the Kushtia district. Among these, 14 were tissue samples (BT-1–14) and six were fecal samples (BF-1–6). We used the mitochondrial gene cytochrome *b*, one of the most abundant and frequently used genetic markers, for polymerase chain reaction amplification and sequencing. Out of the 20 samples, 12 tissue samples and 2 fecal samples were successfully amplified and sequenced. However, two tissue samples and four fecal samples yielded chimeric sequences, rendering them unsuitable for annotation. The sequences of the successfully amplified samples were compared to those deposited in the National Center for Biotechnology Information database using basic local alignment search tool to identify the bat specimen collected. The study identified six different bat species using both morphological and genetic data, which may carriers of the NiV.
**Conclusions:** Our results suggest that additional research should be conducted to gather more information on fruit bats from different localities across the country. The study contributes to the establishment of appropriate measures for NiV carrying disease control and management.

**Keywords:** cytochrome *b*, ecology, Nipah virus, *Pteropus*, zoonotic

## Introduction

Fruit bat species play distinct ecological roles (Chan et al. 2021), including as pollinators and seed dispersers. The accurate species identification helps assess the ecological functions of bats perform within the selected ecosystems (Kasso and Balakrishnan 2013). However, fruit bats (i.e., *Pteropus* bats) are the primary reservoir of the zoonotic virus, Nipah virus (NiV) (Skowron et al. 2022). Despite their ecological importance, there has been limited research on the occurrence, diversity, and richness of bats in the Bangladesh (Olival et al. 2020; Ul Hasan and Kingston 2022). According to International Union for Conservation of Nature (IUCN) 2023 (Schmidt et al. 2023), a total of 160 mammal species were identified to exist in Bangladesh, however, only 31 of these were confirmed as bat species, representing 8 different families. Among these, 22 species were voucher specimens (physical specimens preserved for reference), and 9 were recorded based on photographs (Hassan et al. 2020; Ul Hasan and Kingston 2022). Many bat species have not been identified or have been misidentified or have doubtful occurrences in the country (Ul Hasan and Kingston 2022). However, the use of morphological evidence to identify bat species remains challenging as few bat species have easily distinguishable features that allow for accurate identification based solely on morphology without additional data, such as molecular analysis (Ul Hasan and Kingston 2022). Relying solely on photographic or morphological evidence without authentication can compromise the scientific credibility of species records

(Aguiar et al. 2017; Rogers et al. 2017). Therefore, more rigorous and comprehensive research, including molecular identification methods (Gager et al. 2016), is required to improve our understanding of bat diversity and distribution in Bangladesh.

Molecular identification, including DNA barcodes, enable the identification of cryptic species and provide insights into the genetic diversity within populations, aiding in more accurate biodiversity assessments (Francis et al. 2010). Genetic identification using cytochrome *b* (*cytb*) primers is emphasized as an efficient, specific, and reliable method for identifying autochthonous bats (Lim et al. 2004; Pulvers and Colgan 2007). A study demonstrates the efficacy of using *cytb* polymerase chain reaction (PCR) on bat faecal specimens for specific identification (Arnaout et al. 2022; Lim et al. 2004). However, the molecular identification of fruit bat species in Bangladesh has been underexplored (Olival et al. 2020; Rahman et al. 2021). Rigorous surveys are required to fully document and characterize bat diversity and identification in Bangladesh. Such surveys are essential for conservation efforts and gaining a better understanding of the country's biodiversity (Hassan et al. 2020).

Genetic and taxonomic analyses of bat populations provide a comprehensive understanding of bat evolution and species diversity. The analysis helps resolve taxonomic controversies and identify cryptic species that may be morphologically similar but genetically distinct (Baird et al. 2017; Camacho et al. 2022). The *cytb* gene is a marker used to identify species-level phylogenies in mammals, including bats, and has been used in epidemiological monitoring and investigation (Caraballo et al. 2020). Understanding the diversity of bats in Bangladesh can help formulate effective conservation strategies (Zhang et al. 2022).

According to the Institute of Epidemiology Disease Control and Research, 325 NiV cases were reported in Bangladesh from 2001 to 2022, with more than 230 deaths (> 70% case fatality rate) (Bangladesh 2023). According to a statement made by the health ministry, NiV outbreaks have been reported in Meherpur in 2001 (Kulkarni et al. 2013) and in the Kushtia district in 2007 (Homaira et al. 2010). Howeverr, urban development, deforestation, and anthropization have cause an overabundance of bats in cities, which facilitates easy contact with other species and the creates a new zoonotic epidemics that pose risk to people (Dimkić et al. 2021; Jung and Threlfall 2016). This study was conducted in the Kushtia district (Nipah belt) of Bangladesh to analyze Bangladeshi fruit bats. Morphological and molecular techniques were used to identify and classify various fruit bat specimens. This study provides valuable insights into the evolutionary relationships, identification, and diversity of bats in Bangladesh, particularly those that may carry NiV.

## Materials and Methods

### Study design and specimen collection

The survey area for collecting bat samples was selected in various regions of the Kushtia district, including three upazillas (Kushtia Sadar, Mirpur, and Bheramara) (Fig. 1, Table 1). In total, 14 bat tissue (wing or ear) samples and 6 fecal samples were collected from 3 different places in Kushtia district. Bat tissue samples were collected from dead specimens found in the orchard, specifically in the litchi gardens, or net-captured individuals. The average humidity and temperature data were collected nearby meteorological stations in Bangladesh (Liu et al. 2023). Among the samples, 75% (n = 15) were collected during summer season (mid-March to mid-June, 2021–2022). All the samples were stored in the laboratory refrigerator at –20°C temperature.

For fecal sample collection, a cleaned polythene sheet was spread under the roosting tree, and freshly excreted

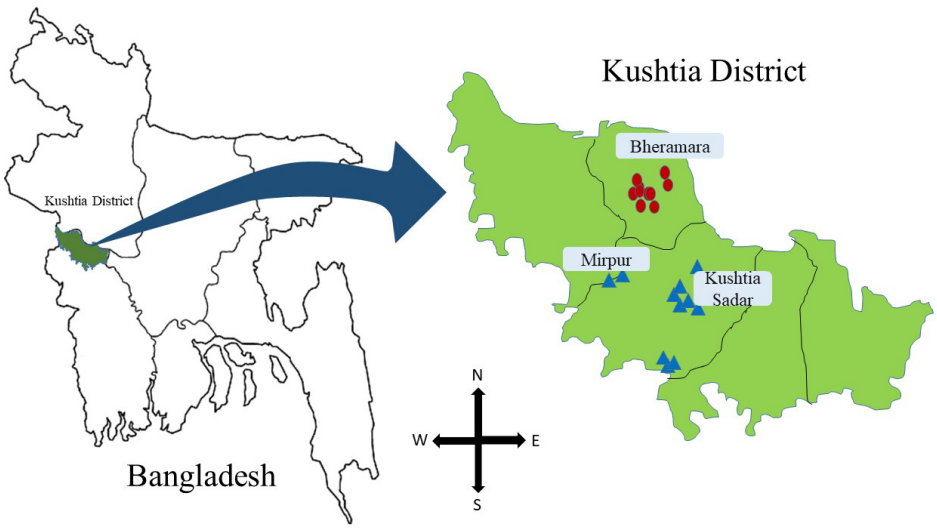


Fig. 1 Sites of sample collection and bat survey regions in the Kushtia district's at three upazilas (Bheramara, Mirpur, and Kushta upazila). Map of the Kushtia district on the left; color-coded three places on the right. The red-colored marked circular indicates Bherameara on the right, while blue-colored triangle pyramid-shape regions indicate Mirpur (blue color) and Kushtia sadar (navy color).

**Table 1** A detail sample information included types, collection locality, and their geographic information

| Sample type | S. no. | Sample code | Locality code | Collection locality | Latitude | Longitude | Date | Temperature (°C) | Humidity (%) |
|---|---|---|---|---|---|---|---|---|---|
| Tissue sample | 1 | BT-1 | KS-1 | Kushtia Sadar | 23.9037 | 89.1200 | 24/03/2021 | 27 ± 0.24 | 76 |
| | 2 | BT-2 | BR-2 | Bheramara | 24.0428 | 88.9682 | 20/05/2021 | 38 ± 0.69 | 76 |
| | 3 | BT-3 | BR-3 | Bheramara | 24.0428 | 88.9682 | 20/05/2021 | 38 ± 0.73 | 55 |
| | 4 | BT-4 | BR-4 | Bheramara | 24.0428 | 88.9683 | 20/05/2021 | 38 ± 0.82 | 55 |
| | 5 | BT-5 | BR-5 | Bheramara | 24.0427 | 88.9682 | 20/05/2021 | 38 ± 0.93 | 53 |
| | 6 | BT-6 | BR-6 | Bheramara | 24.0341 | 88.9833 | 21/05/2021 | 29 ± 0.25 | 66 |
| | 7 | BT-7 | BR-7 | Bheramara | 24.0341 | 88.9833 | 21/05/2021 | 29 ± 0.12 | 66 |
| | 8 | BT-8 | BR-8 | Bheramara | 24.0438 | 88.9667 | 03/06/2021 | 37 ± 1.20 | 55 |
| | 9 | BT-9 | KS-2 | Kushtia Sadar | 23.9101 | 89.1251 | 14/06/2021 | 34 ± 0.56 | 55 |
| | 10 | BT-10 | IU-1 | Islamic University, Kushtia Sadar | 23.7234 | 89.1510 | 04/10/2021 | 28 ± 0.73 | 55 |
| | 11 | BT-11 | BR-10 | Bheramara | 24.0432 | 88.9681 | 14/04/2022 | 29 ± 0.49 | 55 |
| | 12 | BT-12 | BR-11 | Bheramara | 24.0431 | 88.9677 | 14/04/2022 | 29 ± 0.38 | 86 |
| | 13 | BT-13 | IK-2 | Islamic University, Kushtia Sadar | 23.7222 | 89.1509 | 22/03/2022 | 25 ± 0.62 | 86 |
| | 14 | BT-14 | IU-3 | Islamic University, Kushtia Sadar | 23.7239 | 89.1487 | 22/02/2022 | 23 ± 0.52 | 73 |
| Fecal sample | 1 | BF-1 | KS-1 | Kushtia Sadar | 23.9037 | 89.1200 | 08/03/2021 | 32 ± 0.49 | 67 |
| | 2 | BF-2 | BR-2 | Kushtia Sadar | 23.9037 | 89.1200 | 08/03/2021 | 32 ± 0.27 | 62 |
| | 3 | BF-3 | BR-3 | Kushtia Sadar | 23.9038 | 89.1199 | 22/03/2022 | 25 ± 0.34 | 51 |
| | 4 | BF-4 | BR-4 | Mirpur, Kushtia | 23.9350 | 88.9963 | 22/03/2022 | 25 ± 0.89 | 52 |
| | 5 | BF-5 | BR-5 | Mirpur, Kushtia | 23.9446 | 89.0020 | 08/03/2022 | 26 ± 0.23 | 58 |
| | 6 | BF-6 | BR-6 | Bheramara | 24.0432 | 88.9681 | 14/04/2022 | 29 ± 0.87 | 49 |

Values are presented as mean ± standard deviation.


**Fig. 2** (A-D) The representative bat images from the samples collected in Kushtia. (A) Greater false vampire bat (*Megaderma lira*), (B) short nosted fruit bat (*Cynopterus sphinx*), (C) a flying fox bat (*Pteropus giganteus*) in a orchard garden, and (D) a flying fox bat (*P. giganteus*) in a mist net.

waste was collected the following morning. Targeted bats were collected using mist and hand nets from various habitats including orchards, nearby agricultural areas, and other roosts in different areas. The GPS coordinates of the bat specimens collection sites were recorded along with the corresponding habitat types. After the collection of fecal material and tissue specimens, living bats were released. If specimens were identified, the voucher specimens were preserved in the central laboratory of the Biological faculty at Islamic University, Kushtia 7003, Bangladesh. All morphological characteristics and body weight were measured in the field. Furthermore, certain tissue specimens were collected from dead bats found hanging from an electric line, accidentally wounded or dried (Fig. 2).

Tissue and fecal samples of bats were collected from 3 different regions of the NiV-prone area of Kushtia. On the date of sampling, we carefully determined the size of the collected feces by measuring it with a ruler and also observed the shape and color of the bat feces considered for identification (Rahman et al. 2022). Droppings of different colors were defined on each collection sheet, with each color representing a distinct dietary component. The bat tag with number from each sampled bat and the corresponding fecal samples were counted separately. A representative image of the bat samples collected in Kushtia is provided in Figure S1.

## Morphological identification

Taxonomic phenotypic haracteristics (such as size, weight, and color) for morphological identification were assessed

as described by Neaves et al. (2018). The external morphology of the animal was examined, which included features such as pelage (fur) color. After collecting the necessary data and tissue samples, the animal/tissue is fixed in formaldehyde and then stored in 75% ethanol.

## Molecular identification

***DNA extraction and polymerase chain reaction amplification***

Genomic DNA was extracted from tissue using the chemagic viral NA/gDNA kit (PerkinElmer, Waltham, MA, USA) on a Chemagic 360 instrument (PerkinElmer) and DNA from fecal samples was extracted using the QIAamp Fast DNA stool Mini Kit with with Bead beatingprocess followingthe manufacturer's protocol guidelines.

The extracted DNA was used as a template for PCR amplification with primer set *cytb* F: (5′-GAGGMCAAATAT CATTCTGAGG-3′) and *cytb* R: (5′-TAGGGCVAGGACT CCTCCTAGT-3′) targeting the partial mitochondrial *cytb* (Gyawali et al. 2019). The amplification reactions were performed in a total volume of 25 $\mu$L and included master mix (1× Invitrogen Platinum Taq Buffer, 0.25 mM each of deoxynucleotide triphosphate, 2.0 mM $MgCl_2$, and 0.5 units of Taq DNA polymerase), 10 pmol of each primer, 100 ng of genomic DNA. The reactions were conducted using a PTC-200 DNA Engine Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: initial denaturation at 95°C for 6 minutes; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 40 seconds; and final elongation at 72°C for 10 minutes followed by a hold at 4°C (Fig. S2). To ensure that the reactions yielded adequate amplicon sizes, PCR products were electrophoresed and visualized on 1.0% agarose gels containing ethidium bromide (10%). Sequencing was performed by the Virology Laboratory, International Center for Diarrhoeal Disease Research, Bangladesh (icddr,b), Mohakhali, Dhaka Bangladesh, following the manufacturer's instructions.

***Nucleotide sequencing and sequence similarity search***

Nucleotide sequencing was carried out in an automated ABI3500 XL Genetic Analyzer (Applied Biosystem, Foster City, CA, USA) and Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem), as per kit protocol. Sequencing was performed by Virology Laboratory, icddr,b Mohakhali, Dhaka Bangladesh, according to the manufacturer's instructions. For species identification of the captured samples, sequence similarity searches were performed using the basic local alignment search tool (BLAST) server (http://www.ncbi.nlm.nih.gov/BLAST/), National Center for Biotechnology Information (NCBI) (National Institutes of Health, Bethesda, MD, USA).

## Phylogenetic analysis

The sequences were aligned using the CLUSTAL module in MEGA 11. Phylogenetic analysis was performed using neighbour-joining (NJ) and maximum likelihood (ML) method with *cytb* sequences in MEGA 11 (version 11.0.7). In addition, ML analysis was conducted using the Hasegawa–Kishino–Yano substitution model with gamma + invariant. The branch support of the NJ/ML tree was assessed using bootstrap analysis (1,000 replications) (Hernández- Dávila et al. 2012). In this analysis, we used 62 sequences, including 14 bat sequences, acquired from NCBI (Table S1). The analysis used 447 aligned base pairs from *cytb*. The final phylogenetic tree was presented using Interactive Tree of Life (iTOL) software (Letunic and Bork 2021).

# Results

## Morphological identification

Among 20 individuals from the 3 localities (Kushtia Sadar, Mirpur, and Bheramara), we identified 4 different species based on distinct morphological characteristics: A) greater false vampire bat (*Megaderma lyra*, Megadermatidae), 5.9–6.4 cm in body length, 28.96–33.72 g in weight, and morphological features of large ears and no tail, and blue-gray fur overall with a brownish-gray underside; B) short-nosed Indian fruit bat (*Cynopterus sphinx*), 6.8–8.6 cm in body length, 56.9–74.23 g in weight, with morphological features of bright orange or yellowish-brown coloring and darker ventral fur than dorsal fur; C) *Pteropus giganteus* bat captured in a orchard garden; and D) Indian flying fox (*P. giganteus*, Pteropodidae): 23–26 cm in body length, 1.4–1.5 kg in weight, with the morphological features of large black wings, they captured in a mist net (Table 1, Fig. 2).

## Molecular identification and phylogenetic relationship

Out of the collected 20 samples, 2 tissue samples (BT-5 and -9) and 4 fecal samples (BF-2, -3, -4, and -5) could not be identified because of their chimeric sequences and low DNA quality (Tables 1, 2). Thus, we obtained *cytb* sequences from 14 samples including 12 tissue samples and 2 fecal samples (BF-1 and BF-6), ranging from 420–447 bp in length. BLAST server (NCBI) analysis showed the *cytb* sequences were an 82% (BF-6) to 100% (BT-6, BT-14, BF-1) match those of 6 bat species with 100% sequence coverage (Table 1 and Table S2).

The samples BT-1 (OP856817), and BF-1(OP856810) showed more than 99% similar to *P. giganteus* while BT-14 (OP856822) matched to the same clade with *Pteropus lylei cytb* sequence. Furthermore, BT-2 (OP856812), BT-4 (OP856811), BT-6 (OP856814) and BT-7 (OP856815) *cytb* gene sequence were more than 99% similar to those from *C.*

**Table 2** Information on the bats locality, codes, morphological, and molecular features

| Sample type | Sample code | Locality code | Collection locality | Identified species | Morphological identification | | | Molecular identification | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Body length (cm)/ fecal size/pile | Body weight (g)/ fecal weight (g) | Morphological characters | Success or fail | BLAST coverage (%) | BLAST identity (%) |
| Tissue sample | BT-1 | KS-1 | Kushtia Sadar | *Pteropus giganteus* | 24.1 | 432.0 | Dark brown to black fur color, fox-like appearance of face | O | 100 | 99 |
| | BT-2 | BR-2 | Bheramara | *Cynopterus sphinx* | 6.8 | 59.8 | Yellowish-brown in color, short nose | O | 100 | 99 |
| | BT-3 | BR-3 | Bheramara | *Rousettus leschenaultii* | 11.2 | 106.0 | Brown to grey-brown in color with lighter underparts/reddish-brown in color, large dark eyes | O | 100 | 99 |
| | BT-4 | BR-4 | Bheramara | *C. sphinx* | 8.6 | 74.2 | Yellowish-brown in color, short nose | O | 100 | 99 |
| | BT-5 | BR-5 | Bheramara | Undetermined | 8.1 | 69.2 | Yellowish-brown in color, short nose | X | X | X |
| | BT-6 | BR-6 | Bheramara | *C. sphinx* | 7.9 | 63.7 | Yellowish-brown in color, short nose | O | 100 | 100 |
| | BT-7 | BR-7 | Bheramara | *C. sphinx* | 7.1 | 56.1 | Yellowish-brown in color, short nose | O | 100 | 99 |
| | BT-8 | BR-8 | Bheramara | *Megaderma lyra* | 5.1 | 24.1 | Gray-brown in color, distinctive noseleaf | O | 100 | 99 |
| | BT-9 | KS-2 | Kushtia Sadar | Undetermined | 21.9 | 519.6 | Dark brown to black fur color, fox-like appearance of face | X | X | X |
| | BT-10 | IU-1 | Islamic University, Kushtia Sadar | *Taphozous* spp. | 6.3 | 27.9 | Gray-brown in color, small head | O | 100 | 85 |
| | BT-11 | BR-10 | Bheramara | *Megaderma* spp. | 6.4 | 33.7 | Gray-brown in color, distinctive noseleaf | O | 100 | 89 |
| | BT-12 | BR-11 | Bheramara | *Megaderma* spp. | 5.9 | 29.0 | Gray-brown in color, distinctive noseleaf | O | 100 | 89 |
| | BT-13 | IK-2 | Islamic University, Kushtia Sadar | *Scotophilus kuhlii* | 4.3 | 4.6 | Gray color, pointed teeth | O | 100 | 99 |
| | BT-14 | IU-3 | Islamic University, Kushtia Sadar | *Pteropus* spp. | 27.4 | 497.3 | Dark brown to black fur color, fox-like appearance of face | O | 100 | 100 |
| Fecal sample | BF-1 | KS-3 | Kushtia Sadar | *P. giganteus* | Large pile in a collection clean sheet | ~150 | Cylindrical paste like faecal, black color | O | 100 | 100 |
| | BF-2 | KS-4 | Kushtia Sadar | Unidentified | Small size of fecal pile | ~50 | Pellet-like faecal, light black color | X | X | X |
| | BF-3 | KS-5 | Kushtia Sadar | Unidentified | Small size of fecal pile | ~40 | Pellet-like faecal, black color | X | X | X |
| | BF-4 | MK-1 | Mirpur, Kushtia | Unidentified | Small size of fecal pile | ~50 | Pellet -like faecal, gray-black color | X | X | X |
| | BF-5 | MK-2 | Mirpur, Kushtia | Unidentified | Small size of fecal pile | ~50 | Pellet -like faecal, brown color | X | X | X |
| | BF-6 | BR-12 | Bheramara | *Megaderma* | Small size of fecal pile | ~20 | Pellet-like faecal, black color | O | 100 | 82 |

BLAST: basic local alignment search tool.

*sphinx* isolates. Sequencing of *cytb* from BT-3 (OP856813) and BT-8 (OP856816) revealed good matches with those of *Rousettus leschenaultii* and *M. lyra* with 99% sequence similarity. Additionally, BT-13 (OP856821) *cytb* also matched with that of *Scotophilus kuhlii* with 99% sequence similarity (Table 2 and Fig. 3).

In case of BT-11 (OP856819) and BT-12 (OP856820), the *cytb* sequences showed similarity to those of *Megaderma* clade, but with relatively low similarity (89%). The *cytb* gene sequences of BT-10 (OP856818) and BF-6 (OP856823) also matched those of *Taphozous* (85% similarity) and *Megaderma* (82% similarity), respectively, with relatively low similarities (Table 2 and Fig. 3). Possibly, these four samples of collected bat (BT-8, -11, -12, and BF-6) are grouped in same genus, *Megaderma*. We could not properly determine the species level with these low similarity of sequences.

BT-1, BT-14, and BF-1 were included in the *P. giganteus* clade. BT-14 was positioned with *P. lylei*, but its species identification was not determined due to incomplete status of the phylogenetic tree, possibly the bat species with *P. lylei.* Moreover, *Pteropus vampyrus* and *P. lylei* are closely related to *P. giganteus,* as established in a previous study (Kunz and Jones 2000). Within this clade, BT-14 formed a sister clade between *P. vampyrus* and *P. lylei*. BT-2, BT-4, BT-6, and BT-7 were all well-grouped in the *C. sphinx* clade. Furtermore, BT-3 was also well positioned within *R. leschenault* clade (Fig. 3).

The identified bats were divided into three groups based on their diet: insectivorous bats (n = 2), frugivorous bats (n = 8), and carnivorous bats (n = 4) (Table 3) (Epstein et al. 2016; Fenton 2001; Islam et al. 2020; Khan 2018; Srinivasulu and Srinivasulu 2002).

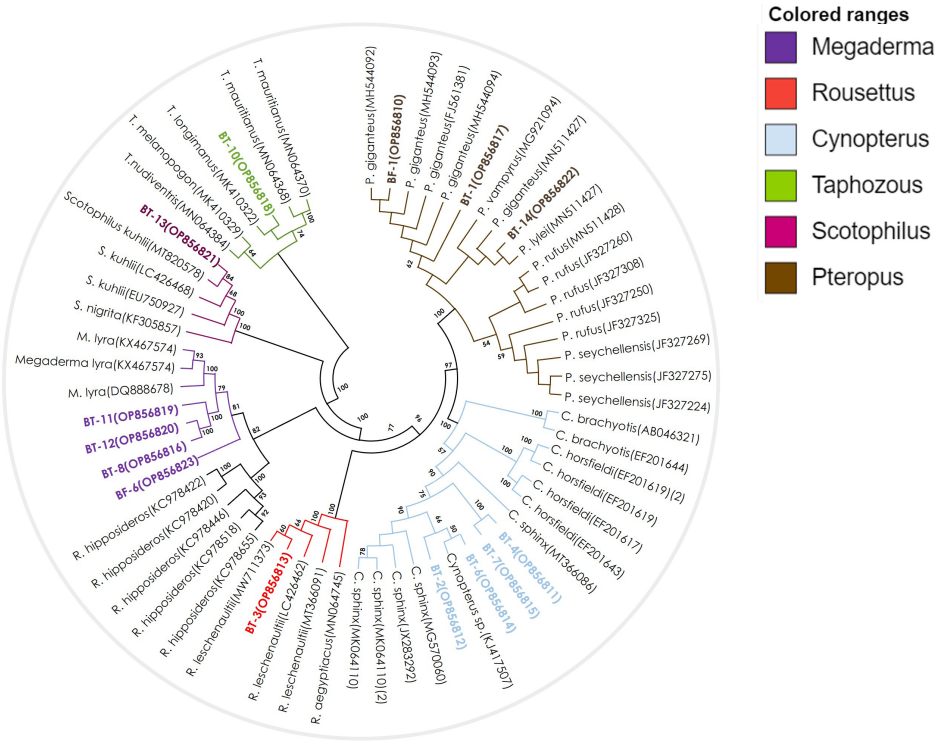


**Fig. 3** The unrooted phylogenetic analysis based on 447 bp of cytochrome c oxidase subunit I (*cytb*) sequences. The reference sequences obtained from National Center for Biotechnology Information and the bat sequneces from this study were used. The sample ID and the parenthesis accession number are provided. Bats from six different genus have been grouped cccording to color. Bootstrap values (> 50%) are provided under each node.

**Table 3** Class of identified bats in this study

| S. no. | Local name (scientific name) | Family | Diet type | Sample code | Number of bat species | References |
|---|---|---|---|---|---|---|
| 1 | Long-winged tomb bat (*Taphozous* spp.) | Emballonuridae | Insectivorous | BT-10 | 1 | Fenton 2001 |
| 2 | Greater false vampire bat (*Megaderma* spp.) | Megadermatidae | Carnivorous | BT-8, -11, -12, BF-6 | 4 | Fenton 2001 |
| 3 | Lesser Asiatic yellow bat (*Scotophilus kuhlii*) | Vespertilionidae | Insectivorous | BT-13 | 1 | Fenton 2001 |
| 4 | Indian flying fox (*Pteropus* spp.) | Pteropodidae | Frugivorous | BT-1, -14, BF-1 | 3 | Epstein et al. 2016 |
| 5 | Greater short-nosed fruit bat (*Cynopterus sphinx*) | Pteropodidae | Frugivorous | BT-2, -4, -6, -7 | 4 | Srinivasulu and Srinivasulu 2002 |
| 6 | Leschenault's rousette (*Rousettus leschenaultii*) | Pteropodidae | Frugivorous | BT-3 | 1 | Islam et al. 2020; Khan 2018 |

## Discussion

In this study, we collected and analyzed bat-guano and -tissues from geographic locations, specifically the Nipah belt region, with a particular emphasis on the Kushtia district in Bangladesh (Fig. 1) and accurately identified six different bat species. The regional interest in understanding the role of bats in viral transmission, as evidenced by a study conducted in West Bengal, neighboring Bangladesh, focused on surveying different bat populations for highly pathogenic viruses, including NiV (Sharma et al. 2019).

Traditionally, species identifcation of African fruit bats has relied on morphological characteristics, including skull shape, dental formula, and unique palatal ridge patterns (Igado and Joannis 2022). In this study, the collected bat samples were initially identified using morphological characteristics, specifically tissue color examination (Fig. 2), employing morphometric data for proper identification of bat species (Gager et al. 2016). The challenges in morphological identification have prompted the adoption of the DNA barcode identifying bat species. DNA barcoding is based on *cytb* obtained from the mitochondrial DNA (mtDNA) genome, which acts as a 'barcode' to identify bat species (Nesi et al. 2011). The mitochondrial-coding *cytb* is regarded as a suitable marker for identifying species, even potentially cryptic species (Mayer et al. 2007). In this study, we performed species identification using sequenced amplified *cytb* and conducted a phylogenetic analysis for confirmation at the species level (Tables 2, 3). Fourteen tissue samples with six bat guano were collected from diverse locations in the Kushtia district, Bangladesh. Nucleotide sequence similarity analysis using BLAST (NCBI) revealed bats belonging to six different genera, classified into three dietary categories: two insectivorous, one carnivorous, and three frugivorous (Table 3) (Epstein et al. 2016; Fenton 2001; Islam et al. 2020; Khan 2018; Srinivasulu and Srinivasulu 2002).

Flying foxes (*P. vampyrus*, formerly *P. giganteus*) and *P. lylei* are genetically closely related. Both species are found in Asia, particularly in India and Bangladesh (Almeida et al. 2014). In this study, results, 21% of the bats were identified as *Pteropus* or megabats (Fig. 4), thought the limitation in determining the proportion of *Pteropus* bats based on the available analysis of samples. A study conducted in Australian study showed a similarity in the percentage of *Pteropus* bats, where 25% of captures were *Pteropus* (Philbey et al. 2008). Additionally, the western Indian Ocean species, includinh *P. vampyrus*, *P. medius*, and *P. lylei*, form a clade with strong support across various studies (Almeida et al. 2014; Chan et al. 2011; O'Brien et al. 2009). Flying foxes are the primary reservoir hosts of NiV (Halpin et al. 2011). Fruit bat species such as *P. giganteus*, *P. lylei*, and *Hipposideros larvatus* have been verified as reservoir hosts in various locations, including Bangladesh, Cambodia, and Thailand (Wacharapluesadee et al. 2005). In Malaysia, a sero-epidemiological study identified four fruit bat species as potential hosts: *P. hypomelanus*, *P. vampyrus*, *C. brachyotis*, and *Eonycteris spelaea*. Additionally, the insectivorous bat *S. kuhlii* has been implicated in NiV transmission (Joshi et al. 2023; Yob et al. 2001). In our study, the insectivorous bats category included, *S. kuhlii* (BT-13, OP856821), as shown in Table 3 (Epstein et al. 2016; Fenton 2001; Islam et al. 2020; Khan 2018; Srinivasulu and Srinivasulu 2002).

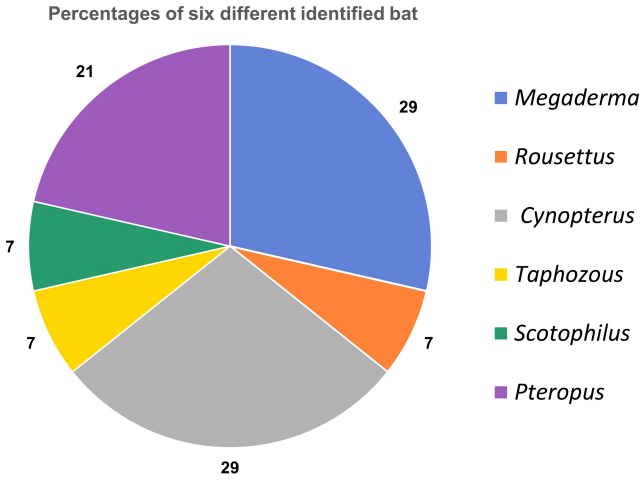


Fig. 4 The pie chart indicates the percentages of each of the six genus of bats that were identified.

Ahmed and Husain (1982) published a checklist of bats in Bangladesh that incuded 52 specimens of 7 species. The species include *P. giganteus* (*P. medius*), *C. sphinx*, *M. lyra* (*Lyroderma lyra*), *S. temmincki* (*S. kuhlii*), and *Tylonycteris pachypus*. In Bangladesh, first *R. leschenaultii* species was confirmed by voucher specimens (Khan 2018). In our study, *R. leschenaultii* was idenfiedd both morphologically and molecularly, as indicated in Figure 3. *R. leschenaultii* is a threatened species according to IUCN red list global assessment (IUCN 2022, Available online: https://www.iucnredlist.org [accessed on 2 January 2023]). Moreover, *R. leschenaultii* has been implicated as a reservoir of filoviruses in Africa (Leroy et al. 2005; Pourrut et al. 2009; Swanepoel et al. 2007) and rotavirus A in Bangladesh (Islam et al. 2020). This species has significant implications for public health and epidemiological research.

The study had a number of limitations including a relatively small number of samples and issues with sequence quality. The presence of *T. longimanchus* in Bangladesh, as recorded by preserved specimens, has been previously noted (Ul Hasan and Kingston 2022). However, there are concerns about its identity, which appears to have low identity (85%) but high coverage (Table 2). This may be result of misidentification, the existence of a cryptic species or lack of available sequence data for the same bat species, which makes it difficult to identify using currently avialbale sequences. Another species, *M. lyra* (identified from fecal

sample, BF-6) is noted for having low similarity (82%) but full sequence coverage (Table 2). This raises questions regarding the accuracy of species identification and the possibility that these bats may represent cryptic species within the *Megaderma* genus. Furthermore, the isolation of DNA from fecal samples present difficulties related to the removal of fibers and undigested particles (Kumar et al. 2016). Phylogeentcic analysis indicated that *M. lyra* and *T. longimanus* are closely related to species within the *Megaderma* and *Taphazous* clade, suggesting potentiak cryptic species. Similarly, related study failed to clarify the *Scotophilus* (Jacobs et al. 2006) and *Rhinolophus* (Stoffberg et al. 2010) bat species identification (owing to cryptic species) with the mitochondrial *cytb* and failed to recover well supported relationships at deeper nodes in the topology. While the study does not directly test for the presence of NiV, it speculates based on previous studies that there may be a potential presence of NiV in these megabat specimens. It underscores the importance of future studies to confirm the reservoir status of megabats for NiV.

## Conclusions

Fruit bats serve as natural reservoirs for the NiV. This study aims to identify potential reservoir species for the NiV within a specific geographic region. Accurate species identification of the associated reservoir bat species is crucial for understanding the dynamics of virus transmission and developing targeted strategies for disease prevention and control. The study identified bats belong to different dietary categories included two insectivorous, one carnivorous, and three frugivorous bats. Alhough their phylogenetic positions have not been properly established, but their current geographic distribution is important in relation to the phylogenetic tree. Molecular data analyses are crucial for shed light on their divergence history. Further research is needed to gather additional data on megabats from various locations and habitats in Bangladesh. The conclusions and observations from research results derived from this research may serve as suggestions for the development of a plan of action in the event of a local epidemic.

## Supplementary Information

Supplementary information accompanies this paper at https://doi.org/10.5141/jee.24.003.

**Table S1.** Information on the National Center for Biotechnology Information-acquired reference accession accession number including seven category of forteen bat species (this study) used for phylogentcic analysis. **Table S2.** The sample information with their sources, sequence similarity, sample identification, and sequence length (accessed on: https://www.ncbi.nlm.nih.gov/nuccore/). **Fig. S1.** Representative image of bat survey area. **Fig. S2.** A graphic presenation of polymerase chain reaction cycles and duration.

### Abbreviations

NiV: Nipah virus
IUCN: International Union for Conservation of Nature
NCBI: National Center for Biotechnology Information
NJ: Neighbour-joining
ML: Maximum likelihood
PCR: Polymerase chain reaction

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### Authors' contributions

MMHF, Md. Mafizur Rahman, HMF, and SJL designed the research for the whole paper. Md. Mafizur Rahman and SJL coordinated the project. WH, YCP, SJL, HMF, MTR, and Md. Mafizur Rahman research conducted. AA, Md. Mafizur Rahman, SJL, HMF, Md. Mahfuzur Rahman, and Md. Mafizur Rahman wrote the original draft preparation. AA, Md. Mahfuzur Rahman, MTR, and WH revised the paper. SJL and HMF annotated the genes. YO, YCP, and Md. Mafizur Rahman conducted data analysis and drafted the paper.

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### Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. The genetic sequence information was provided the following accession numbers (OP856810-OP856823).

### Ethics approval and consent to participate

The study protocol (Protocol No. BAU/2019/65) was approved by the ethical review committee of Bangladesh Agricultural University and the P&D committee of the Department of Biotechnology and Genetic Engineering at Islamic University, Kushtia-7003, Bangladesh.

### Consent for publication

All authors with this manuscript have provided their consent for publication.

### Competing interests

Yung Chul Park, one of co-authors, has been an editor of the *Journal of Ecology and Environment* since 2019; however, he was not involved in the peer reviewer selection, evaluation, or decision process

of this article. The authors declared that they have no other potential conflicts of interest relevant to this article.

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