



# Phylogenetic relationships of a novel bat fly species infesting the geographically widespread Old World fruit bat, *Rousettus leschenaultii*, in Southern Asia

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## Abstract

The global epidemiological significance of bats and their blood-sucking ectoparasites is increasingly recognized. However, relevant data are scarce from Pakistan where the Palearctic and Oriental zoogeographic regions meet. In this study, 200 bats belonging to five species were examined for the presence of ectoparasites in Pakistan. Bat flies were found only on Leschenault's fruit bat (*Rousettus leschenaultii*). The prevalence of infestation did not correlate with habitat type and host traits including age, reproductive status, and sex. All bat flies represented the same *Eucampsipoda* species which was shown to be morphologically different from all species of its genus with known south Asian distribution and belonged to a separate phylogenetic group. These results highlight the existence of a hitherto undescribed bat fly species in southern Asia, which is not shared by the fruit bat species (*R. leschenaultii*) and insectivorous ones (e.g., *Rhinopoma microphyllum*) thus probably playing a role only in intraspecific transmission of pathogens.

**Keywords** Chiroptera · Nycteribiidae · *Eucampsipoda* · COI

## Introduction

Bats (Mammalia: Chiroptera) can be infested with a broad taxonomic range of ectoparasites such as ticks, mites, chiggers, bugs, fleas, and flies (Jones et al. 2009). Some of these parasites also carry disease-causing viruses and bacteria which are of zoonotic importance. Due to the mobility of

bats and their presence in urban settlements and near human dwellings, the epidemiological risks associated with carrying these ectoparasites and vector-borne pathogens is high (Ikeda et al. 2020; Szentiványi et al. 2020; Welbergen et al. 2020).

Most ectoparasites of bats feed on blood, and thus will have access to vector-borne pathogens in bat blood (Loftis et al. 2005; Lei and Olival 2014). Whether these

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ectoparasites are able to transmit bat-borne pathogens towards either domestic or wild animals as well as humans will strongly depend on both the causative agents of relevant diseases, and also on the degree of host specificity of any bat ectoparasites that are their competent biological or mechanical vectors.

Among haematophagous bat ectoparasites, bat flies (Diptera: Streblidae, Nycteribiidae) live on the patagium and in the fur coat of their host (Dick and Patterson 2006). Nycteribiid bat flies belong to three subfamilies, with 12 genera and 275 species which are thought to be strictly host-specific (Dick and Patterson 2006). They are dorsoventrally flattened and wingless, move over the pelage of bats, but females also leave the host for laying a thirds instar larva on the walls of the roosting place (Dick and Patterson 2006).

Pakistan has a rich diversity of bats which are mostly studied with respect to their distribution, ecology, and morphology (Perveen and Rahman 2012; Akhtar et al. 2014). However, only two studies were conducted with emphasis on the diversity of bat ectoparasites in this country. In one study morphological identification of ectoparasites of fruit bats (such as *Pteropus vampyrus*) was carried out in Malakand region of Pakistan (Khan et al. 2020). Additionally, the presence of *Rickettsia honei* in ticks (*Carios vespertilionis*) was confirmed using PCR assays in Bajaur region of Pakistan (Ullah et al. 2019). However, no previous study focused on bat flies (Diptera: Nycteribiidae) in Pakistan. Thus, the aim of this study was to investigate biological and environmental factors associated with significant increases in the prevalence of bat flies infesting a geographically widespread Old World fruit bat species Leschenault's fruit bat (*Rousettus leschenaultii*). It was also within the scope of this study to identify bat fly species morphologically and to analyze their genetic diversity and taxonomic relationships based on mtDNA.

## Materials and methods

### Study area

The present study was conducted at roost sites in two provinces of Pakistan, Khyber Pakhtunkhwa and Punjab. Site I: One roosting site is an abandoned, multi-story fort (known as Sheikhpura Fort) located in Sheikhpura City in Punjab (31.70813° N, 73.99078° E) We sampled bats daily from 5 to 12 pm between 18 and 19 in October 2019. Site II: The other roosting site was a cave-like structure created by an interconnected system of abandoned mining shafts located 20 km from Malakand City in Khyber Pakhtunkhwa (KPK) (34.4897° N, 71.7978° E). We sampled bats daily between 4 and 6 pm between 6 and 10 in April 2019. Site III: Bats were captured from the cracks of a dilapidated brick shed

traditionally used to house livestock. The shed was in Malakand district of Khyber Pakhtunkhwa province (34.07757 N° E71.83435° E), located near to Site I.

### Capturing and identification of bat species

Bat species were captured in-flight as they exited roosting sites using mist nets. In addition, hand nets were used to capture bats in crevices of the walls and roof at Site I. We used bite-proof gloves during capturing and handling of bats. Captured bats were kept in separate cotton bags to prevent cross-contamination of ectoparasites from one bat to another. Individuals were identified based on the only published key for bat species documented in Pakistan (Mahmood-ul-Hasan et al. 2009). Morphological measurements (i.e., forearm length and body mass) and demographic information (i.e., age, sex, and reproductive condition) were recorded.

### Ectoparasite collection and preservation

Whole body surfaces of captured bats (e.g., ears, neck, head, body, patagia) were examined for ectoparasites which were collected using forceps and preserved in 70% ethanol in sterilized, labelled vials. After examination, bats were released at the roosting sites.

### Morphological identification of ectoparasites

Wingless bat flies were identified using a stereomicroscope (Euromex StereoBlue S/N-EU 1870857). Each ectoparasite was identified based on morphological characteristics using published taxonomic keys (Theodor 1967; Maa 1971).

### Molecular identification

Bat flies were crushed with liquid nitrogen and used for genomic DNA extraction by using GeneJET Genomic DNA Purification Kit (GeneJET K0721, GeneJET K072, Thermo Scientific, USA) as directed by the manufacturer. An approx. 710-bp-long fragment of the cytochrome *c* oxidase subunit I (COI) gene was amplified using the primer pair LCO1490 and HCO2198 (Folmer et al. 1994). All PCR reactions were performed in a 25 µl volume, containing 12 µl master mix, 1 µl of each primer, 5 µl DNA template, and 6 µl of double-distilled water. PCR conditions for both reactions consisted of an initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation for 30 s at 95 °C, primer annealing for 60 s at 47 °C, elongation for 30 s at 72 °C, and final elongation for 30 min at 60 °C (Tortosa et al. 2013). The confirmation of PCR products was carried out by gel electrophoresis. Five microliters of amplified products were subjected to 2% agarose gel. The amplified DNA fragments of specific size were visualized by UV light after staining with ethidium bromide. Further, PCR

products were sequenced in one direction only using primer LCO1490 (1ST BASE, Malaysia) and sequences were checked using BioEdit (Version 7.2.5) and EMBOSS Seqret. New sequences were submitted to GenBank (accession numbers: OM283588-OM283593). Phylogenetic tree was constructed after 1000 replicates by the maximum likelihood method, Jukes-Cantor model using MEGA 7.0 (Kumar et al. 2016).

## Results

### Fly infestation of bat species

A total of 200 bats were captured that belonged to 5 species. *Pipistrellus pipistrellus* ( $n=10$ ) was captured at Site III (Animal shed). *Rousettus leschenaultii* ( $n=82$ ), *Scotophilus kuhlii* ( $n=1$ ), and *Pipistrellus javanicus* ( $n=17$ ) were captured at Site I (Castle). *Rousettus leschenaultii* ( $n=42$ ) and *Rhinopoma microphyllum* ( $n=48$ ) were captured at Site II (Cave). Bat flies were only found on *R. leschenaultii*.

### Morphological and molecular-phylogenetic analysis of bat flies

A total of 53 bat flies were collected from bats in Malakand and Sheikhpura. Morphologically, all flies belonged to the same species. This *Eucampsipoda* sp. was morphologically different from all species of its genus with known south Asian distribution, i.e., *Eucampsipoda africana*, *E. inermis*, *E. hyrtlii*, and *E. latisternum* (Supplementary Text 1). Most importantly, there were two long setae dorsally on the abdomen of females which were situated in the middle region (Fig. 1), unlike in the other four species (Supplementary Fig. 1).

Molecularly, the COI sequence OM283588 had only 96.4% (609/632 bp) sequence identity with *E. inermis*

(KF021493) and a similar 96.4–96.8% (609–612/632 bp) identity with *E. africana* (MH151066, LC536588) reported in GenBank. Phylogenetically, all sequences of this novel bat fly species clustered together, in the clade of Asian sequences from Pakistan and the Philippines, and well-separated from the clade of *Eucampsipoda* species from Africa and islands nearby. The phylogenetic group of this bat fly species from Pakistan received moderate bootstrap support (Fig. 2). In summary, based on morphological and molecular-phylogenetic properties, the fly species reported here from fruit bats in Pakistan probably represents a new species.

### Comparison of fly infestation between bat species

The number of fly-infested bats (all *R. leschenaultii*) was 21 at Site I and 15 at Site II (Table 1). The infestation rates were not significantly different according to habitat type and host traits including age, reproductive status, and sex of individuals belonging to this bat species. However, adult bats carried more ectoparasites than juveniles, and females in comparison with males (Table 2). Similarly, the body condition index (BCI) of *R. leschenaultii* showed no significant association with fly infestation (Table 2).

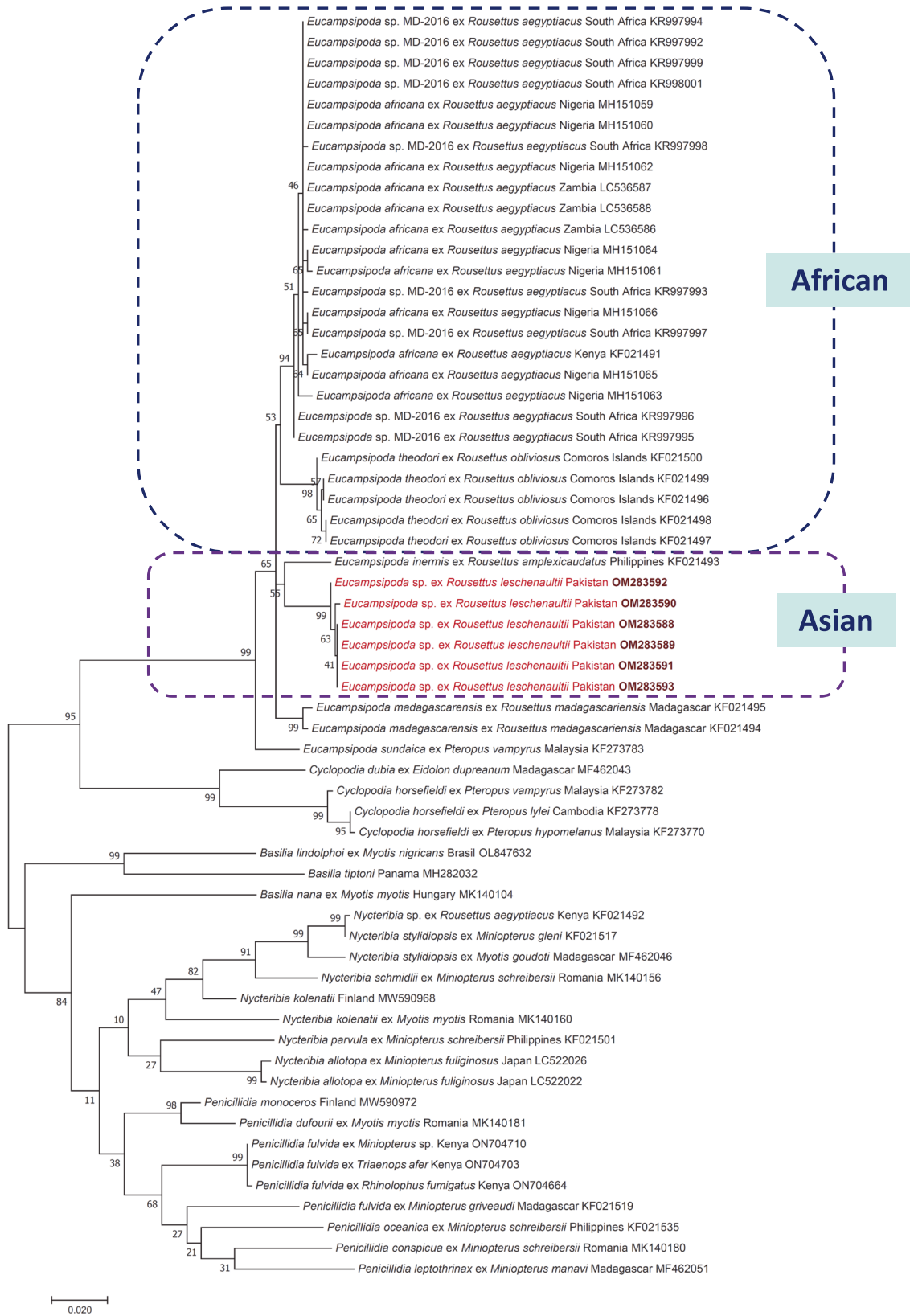
## Discussion

To our knowledge, this is the first morphological and molecular-taxonomic investigation of bat flies from Pakistan and its region. The present study also provides information on the ecology and host preference of wingless bat flies parasitizing the Leschenault's fruit bat (*R. leschenaultii*) from roosting sites in southern Asia, Pakistan.

This species is among the most studied fruit bats in the Old World. In addition to protozoan parasites, *R. leschenaultii*

**Fig. 1** Morphological characters of *Eucampsipoda* sp. collected in Pakistan. **A** Dorsal view of female, showing (1) mid-dorsum without long setae; (2) two long setae situated close to mid-dorsum; (3) tergite no. 6; (4) six long setae posteriorly. **B** Ventral view of male: (1) thorax anteriorly rounded, distance between ctenidia more than half width of thorax; (2) median sternal suture well-marked posteriorly; (3) sternite 1 + 2 relatively long, trapezoidal, with a ctenidium consisting of 36 spines





**Fig. 2** Phylogenetic tree of bat flies based on COI gene sequences. In each row of individual sequences, the host and country of origin and the GenBank accession number are shown after the species name. All sequences from this study are marked with red fonts and maroon accession numbers. The evolutionary history was inferred by using the maximum likelihood method based on the Jukes-Cantor model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 61 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 617 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

and *R. aegyptiacus* are known as carriers of viruses from 19 families and bacteria from at least 13 families (Ramanantsalama et al. 2022). Pakistan occupies a very important zoogeographic region from the point of view of these two fruit bat species because in its territory their range overlap (Ramanantsalama et al. 2022; (Kwiecinski and Griffiths 1999). *Rousettus* spp. were also shown to harbor coronaviruses in India, which neighbors Pakistan (Yadav et al. 2020).

Among the studies carried out in the Afrotropical zoogeographic region, in Gabon, five bat species, including *R. aegyptiacus*, were captured and the total prevalence of nycteribiid bat flies was 38.0%, with 18.7% in case of *E. africana* (Obame-Nkoghe et al. 2016). The most relevant study conducted in the Palearctic zoogeographic region (in the Middle East, Turkey) reported the bat fly *E. hyrtlilii* from *R.*

*aegyptiacus* (Çetin et al. 2020). In addition, *E. inermis* is known to infest Geoffroy's roussette, *R. amplexicaudatus* in southeastern Asia, representing the Oriental zoogeographic region (Tortosa et al. 2013). However, the nycteribiid bat fly species collected from *R. leschenaultii* in Pakistan was shown to differ both morphologically and phylogenetically from all these bat flies, therefore probably represents a new species.

In the bat fly species, *E. africana* viruses and bacteria from several groups of vector-borne pathogens have been identified. These include causative agents of animal diseases (Qiu et al. 2020), as well as zoonotic pathogens, such as orthoreoviruses, orthobunyaviruses (Jansen van Vuren et al. 2016, 2017), and bartonellae (Bai et al. 2011). Although these bat flies are parasites specialized for bats as hosts and bite humans only opportunistically (Wenzel and Tipton 1966; Dick and Patterson 2006; Reeves and Lloyd 2019), they may still play a role in the zoonotic transfer of bartonellae via their excreta (Bai et al. 2011).

Most bats spend their life in a colony. They make roost in specific places such as trees, caves, and even man-made buildings (Jones et al. 2009). *Rousettus leschenaultii* that was found fly-infested in this study also occurs in urban areas (Lučan et al. 2016; Nordin et al. 2021) rendering its high individual number in colonies epidemiologically even more significant.

Morphological characters of fruit bats, such as thicker pelage and fur of their adults, make these favorable hosts for ectoparasites (Jones et al. 2009; Dick and Patterson 2006), while subadult hosts can bear fewer parasite loads than adult ones (Patterson et al. 2007). Several studies revealed that prevalence rates of ectoparasites were associated with sex, age, and extrinsic and intrinsic factors (Bush et al. 1997; Combes 2001;

**Table 1** Bat fly count and prevalence according to bat species, habitat type, age, reproductive status, and sex

Variable	Categories	No. individuals examined	No. with bat flies (prevalence %)	Chi-square	P value
Bat species	<i>Pipistrellus javanicus</i>	17	0	200	<0.01
	<i>Pipistrellus pipistrellus</i>	10	0		
	<i>Rhinopoma microphyllum</i>	48	0		
	<i>Rousettus leschenaultii</i>	124	36 (29.03%)		
	<i>Scotophilus kuhlii</i>	1	0		
Habitat type	Site I (castle)	100	21 (21%)	2.91	0.23
	Site II (cave)	90	15 (15.67%)		
	Site III (animal shed)	10	0		
Age	Adult	149	26 (17.45%)	0.12	0.73
	Juvenile	51	10 (19.6%)		
Reproductive status	Lactating	2	0	3.52	0.47
	Non-breeding (females)	90	12 (13.3%)		
	Non-scrotal	1	0		
	Post-lactating	34	7 (20.58%)		
	Scrotal	73	17 (23.28%)		
Sex	Male	76	17 (22.36%)	1.58	0.21
	Female	124	19 (15.32%)		

**Table 2** Comparison of bat fly count and prevalence with bat species and demographic traits

Bat species	Ectoparasite count	Ectoparasite prevalence (%)	Age		Sex		Reproductive status		BCI
			Adult	Juvenile	Male	Female	Non-reproductive	Reproductive	
<i>Pipistrellus javanicus</i>	0	0	15	2	7	10	9	8	0.12 ± 0.03
<i>Pipistrellus pipistrellus</i>	0	0	10	0	2	8	7	3	0.11 ± 0.04
<i>Rhinopoma microphyllum</i>	0	0	37	11	21	27	20	28	0.66 ± 0.1
<i>Rousettus leschenaultii</i>	53	29.03	60	28	28	60	42	46	0.99 ± 0.21
<i>Scotophilus kuhlii</i>	0	0	1	0	1	0	0	1	0.42 ± 0
<i>P</i> value	< 0.01	< 0.01	0.98		0.44		0.94		< 0.01

Postawa and Nagy 2016). In this study, however, adult bats carried more flies than juveniles and females were more infested than males, although these differences were not significant.

Bat flies usually exhibit a high degree of host-specificity (Dick 2007; Obame-Nkoghe et al. 2016) and most species infest only a single bat species or its phylogenetically close relatives (Fritz 1983; Dick and Patterson 2006). This reflects long co-evolution of bat flies and their hosts which also resulted in immunocompatibility and frequent vertical transfer of bat flies from adult bats to their offspring (Dick and Patterson 2006; Obame-Nkoghe et al. 2016). In particular, within the bat fly genus *Eucampsipoda*, *R. aegyptiacus* is usually infested with either *E. africana* (in Africa: Obame-Nkoghe et al 2016) or *E. hyrtlui* (Middle-East, Turkey: Theodor 1967; Çetin et al. 2020). On the other hand, in south-southeastern Asia *R. leschenaultii* is the host of *E. sundaica*, *E. inermis* (Theodor 1967; Azhar et al. 2015; Samoh et al. 2021) and *E. latisternum* (Morse et al. 2012).

Based on the above, it is highly relevant to *Eucampsipoda* sp. bat flies that high host specificity will diminish their role in interspecific transfer of bat-borne diseases and pathogens (Dick and Patterson 2006). In the present study it was shown that the discovered *Eucampsipoda* sp. infests *R. leschenaultii*. Another important Old World fruit bat, *R. aegyptiacus*, also occurs in Pakistan (Attaullah et al. 2022); therefore, it will be very important to investigate if the newly discovered bat fly species infests both or not, thus influencing pathogen transfer between these two geographically widespread fruit bat species which have parapatric occurrence in Pakistan.

In conclusion, the existence of a hitherto unknown bat fly species was revealed in southern Asia, Pakistan. This blood-sucking ectoparasite is not shared by the fruit bat species *R. leschenaultii* and insectivorous bats of the region.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00436-023-07909-0>.

**Author contribution** AU, SA, SH, KP, JK, and KO performed the statistical analyses, evaluated the results and drafted the paper. AU, SA, AJ, MI, and TK participated in the conception and design of the study. AU, SA, KP, JK, and KO contributed to analyzing data. All authors have read and approved the final manuscript.

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**Data availability** The data presented in this study are available within the article.

## Declarations

**Ethical statement** This study was carried according to instructions of Animal Ethics Committee of the University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan (No. 120/IRC/BMR; Dated 14/04/2021).

**Consent for participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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