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Morphological and genomic characterization of sand flies (Diptera: Psychodidae) with different taxonomic features from three districts in Sri Lanka and the report of a novel *Sergentomyia* species

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ABSTRACT

Objective: To characterize morphologically distinct sand flies collected from three districts in Sri Lanka using integrated morphological and molecular approaches.

Methods: Sand fly specimens received by the Department of Parasitology, University of Kelaniya, Sri Lanka, between January and December 2024, were examined. Specimens with typical morphological traits were mounted for microscopic evaluation, and molecular confirmation was performed through DNA barcoding of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene, followed by sequencing and BLAST analysis.

Results: Of 58 sand flies analyzed, five known species were identified: *Phlebotomus argentipes* (*n*=21; 36.2%), *Sergentomyia (S.) zeylanica* (*n*=21; 36.2%), *S. indica* (*n*=1; 1.7%), *S. bailyi* (*n*=4; 6.9%), and *S. babu insularis* (*n*=4; 6.9%). Two morphologically distinct species were also recorded. Molecular analysis confirmed the first report of *Idiophlebotomus* genus in Sri Lanka (*n*=5, 8.6%). Additionally, a novel *Sergentomyia* species from the Anuradhapura district was identified and described herein as *S. nayanasis* sp. nov.

Conclusions: This study provides the first evidence of *Idiophlebotomus* sp. in Sri Lanka and describes *S. nayanasis* sp. nov., expanding the known phlebotomine fauna of the island. These results highlight the need for continued surveillance, ecological assessments, and vector competence studies to evaluate the potential epidemiological significance of newly detected sand fly species in leishmaniasis-endemic regions.

KEYWORDS: Sand fly taxonomy; Molecular barcoding; Cryptic species; Leishmaniasis vectors

1. Introduction

Leishmaniasis is a neglected tropical disease caused by parasitic protozoans of the genus *Leishmania*, transmitted to humans through the bite of female sand flies, and ranks among the most burdensome vector-borne parasitic diseases globally. According to the World Health Organization, an estimated 700 000 to 1 million new cases occur annually, with visceral leishmaniasis alone responsible for

Summary

Question: What are the morphological and genomic characteristics of morphologically distinct sand flies collected in Sri Lanka ?

Findings: A total of 58 sand flies were characterized using morphological examination and *COI* gene sequencing. Five known species were identified, and molecular analyses confirmed the first record of the genus *Idiophlebotomus* in Sri Lanka and the presence of a novel species, *Sergentomyia nayanasis* sp. nov.

Meaning: These findings expand the known sand fly fauna of Sri Lanka and demonstrate the value of integrated morphological and molecular approaches for detecting previously unrecorded and novel taxa.

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tens of thousands of deaths each year, predominantly in low- and middle-income countries across Asia, Africa, and Latin America[1]. The disease manifests across a clinical spectrum ranging from self-healing cutaneous lesions to fatal visceral involvement, depending on the infecting *Leishmania* species and the immunological status of the host. Despite this enormous public health burden, the entomological component of leishmaniasis, the identity, diversity, and ecology of its sand fly vectors, remains comparatively understudied, particularly in the Oriental region[2]. Globally, more than 1 000 sand fly species/subspecies have been recognized[2], and only 10% of sand flies have been considered proven or suspected vectors of leishmaniasis[2,3]. The Atlantic Ocean was among the major biogeographical barriers that isolated sand fly species between the "Old World" (the Palearctic, Afrotropical, Oriental, and Australasian regions) and the "New World" (the Nearctic and Neotropical regions)[4]. According to the latest checklist of Phlebotomine sand flies in the world, a total of 17 genera (*Australophlebotomus, Chinius, Demeillonius, Grassomyia, Idiophlebotomus, Libanophlebotomus, Libanophlebotomites, Mesophlebotomites, Paleomyia, Parvidens, Phlebotoiella, Phlebotomiella, Phlebotomites, Phlebotomus, Sergentomyia, Spelaeomyia, Spelaeophlebotomus*) under the Old World have been classified[5]. *Phlebotomus* species are most prevalent in Asia, Africa, and Europe, while *Sergentomyia* species have a wide distribution throughout Africa and Asia[6].

As an island nation, Sri Lanka is located on the Indo-Asian tectonic plate and lies just off the southeastern coast of India. The Palk Strait and the Gulf of Mannar isolate Sri Lanka by acting as natural biogeographic barriers[7]. Along with this isolation and the country's significant topography and climatic diversity, this island has been designated as one of the world's biodiversity hotspots, with endemic fauna and flora[8,9]. Paradoxically, despite being a recognized biodiversity hotspot, Sri Lanka has emerged as a key focus of leishmaniasis in South Asia. Reported cases have risen markedly over the past two decades, and the disease is caused by *Leishmania* (*L.*) *donovani*, a species responsible for fatal visceral leishmaniasis across much of Asia, but which manifests almost exclusively as cutaneous leishmaniasis in Sri Lanka, an epidemiological anomaly that emphasizes the pressing need to better characterize the local vector fauna and transmission dynamics[10]. The first formal record of sand fly taxonomy in Sri Lanka dates back to 1910[11], and since then, several researchers have been involved in sand fly identification and taxonomic characterization[12-16].

According to the most recent checklist by Wijerathna and Gunathilaka, 22 sand fly species have been recorded in Sri Lanka to date, belonging to the genera *Phlebotomus* and *Sergentomyia*[16]. The genus *Phlebotomus* is represented by three subgenera: *Anaphlebotomus*, *Euphlebotomus*, and *Phlebotomus*, which includes four species: *Phlebotomus (P.) stantoni*, *P. argentipes*, *P. marginatus*, and *P. salehi* Mesghali. The genus *Sergentomyia*, comprising 16 known species, is divided into four subgenera: *Grassomyia*, *Neophlebotomus*, *Parrotomyia*, and *Sergentomyia*[16], which are more diverse and widely distributed in Sri Lanka[14,15,17]. Among them, only *P. argentipes glaucus* has been confirmed as a vector responsible for disease transmission[18], although members of the genus *Sergentomyia* are considered non-vectors; this classification has increasingly been challenged by evidence of natural *Leishmania* infections in some *Sergentomyia* species, and their potential bridge vector role in certain transmission foci[19], and their possible role in disease ecology is still being explored in some regions[20]. However, taxonomic studies of sand flies are very limited in the country, as no morphological keys for sand fly species in Sri Lanka were developed until 2020.

There have been advances in sand fly taxonomy, followed by the development of a pictorial key for sand flies in Sri Lanka, and some morphologically distinct species have been recorded in some parts of the country[21]. This further highlights the availability of unrecorded species in the country, with varying degrees of genetic isolation. A fundamental challenge underlying this taxonomic gap is that morphological keys alone are insufficient for reliably resolving species boundaries in phlebotomine sand flies, particularly within speciose groups such as *Sergentomyia*, where convergent morphological traits, intraspecific variation, and high levels of sympatry frequently confound identification[3,14]. Molecular barcoding of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene has emerged as a powerful complement to morphological taxonomy, enabling the detection of cryptic diversity and the objective delimitation of species boundaries in sand flies across tropical regions[22]. Adopting this integrative approach is therefore not merely advantageous but essential for producing a reliable, actionable species inventory in a biodiversity-rich island such as Sri Lanka. Therefore, the objective of this study was to characterize the sand flies reported from different regions in the country that are not compatible with existing taxonomic features using molecular and morphological verification. During the survey, a new country record was established with the identification of the genus *Idiophlebotomus*, and a novel sand fly species was encountered and is herein described as *S. nayanasis* sp. nov.

2. Methods

2.1. Study setup and design

Department of Parasitology, Faculty of Medicine, University of Kelaniya, Sri Lanka, has been a referral center for diagnosing leishmaniasis and identifying disease vectors since 2018. The Department regularly receives sand fly samples from various regions in Sri Lanka for species-level identification. The samples received

during January to December 2024 from three districts (Figure 1) that raised doubts about morphological identification, due to discrepancies in taxonomic features compared with the existing taxonomy for locally reported sand flies, were included in this study.

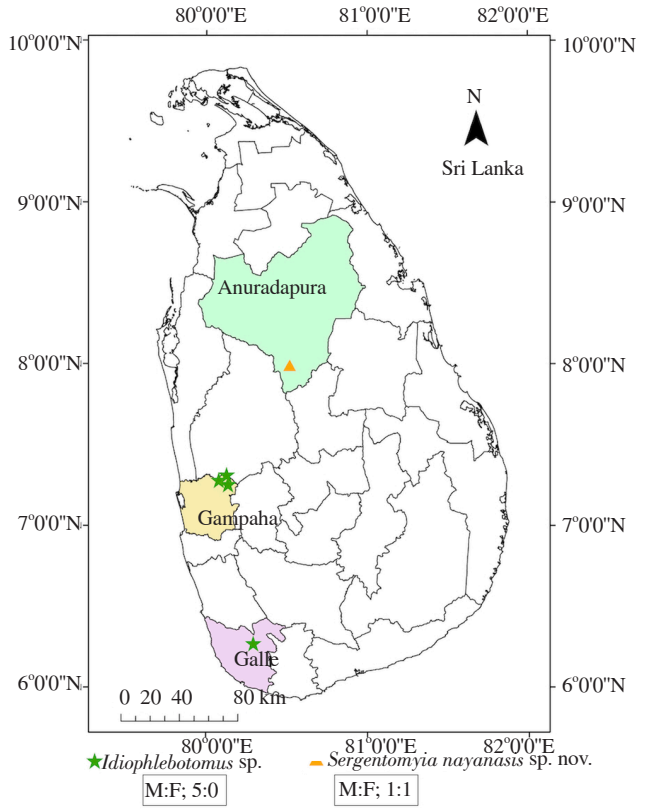

Figure 1. Sand fly specimen collection areas in Gampaha, Galle and Anuradhapura districts along with species composition.

2.2. Specimen processing and morphological identification

Sand fly specimens were immobilized using cold shock by placing the collection tubes on ice for a short period, and then transferred to tubes containing 70% ethanol. Samples were dissected on a glass slide held under a stereomicroscope (Labomed CZM4 Binocular Zoom Microscope, Lobo American Inc., USA, 10× to 40×) for sex determination and separated accordingly. Specimens were further dissected with a drop of saline, separating the head and the terminal part of the abdomen, and mounted with Hoyer's medium. The rest of the body (thorax and abdomen) was preserved at -18 ℃ for molecular characterisation.

Species-level identification was performed under a compound microscope (Model CX33RTFS2) (×100 and ×400) with reference to standard taxonomic keys[16,23,24]. Some specimens in the collection did not match the characteristics of the species reported in the published literature or in the available taxonomic keys for identifying the Sri Lankan sand fly fauna. Identification features of these specimens were captured using a camera (Scope Image 9.0 HDCE-X5) mounted over the same compound microscope, and morphometric measurements were obtained using a binocular microscope aided with a graticule (Table 1). Nomenclature was performed following the guidelines provided by the International Code of Zoological Nomenclature (ICZN). The female specimen is designated as the holotype of *S. nayanasis* sp. nov., and the male specimen is designated as the paratype of *S. nayanasis* sp. nov.

2.3. Molecular identification

2.3.1. Genomic DNA extraction and sequencing

Morphologically identified sand fly specimens were subjected to molecular confirmation using DNA-based techniques. Genomic DNA was extracted using the MightyPrep Reagent for DNA (Takara Bio Inc., Japan) following the manufacturer's protocol. Individual specimens were transferred to 1.5 mL microcentrifuge tubes, and 100 μL of MightyPrep reagent was added. The samples were vortexed thoroughly to ensure complete homogenization, then incubated at 95 ℃ for 10 minutes to facilitate cell lysis and DNA release. The lysates were subsequently centrifuged at 13 000 rpm for 2 minutes, and the supernatant was carefully transferred to a new 1.5 mL microcentrifuge tube as the DNA template for downstream applications.

For species-level identification, DNA barcoding of the mitochondrial *COI* gene was performed using universal primers: LCO1490 (Forward: 5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (Reverse: 5′-AAACTTCAGGGTGACCAAAAAA TCA-3′), targeting an approximately 650 base pair (bp) conserved region.

Polymerase chain reaction (PCR) was carried out in a final reaction volume of 25 μL, consisting of 12.5 μL of Promega® GoTaq Green Master Mix® (2X), 0.25 μL each of forward and reverse primers (10 μM), 2 μL of template DNA (~50 ng/μL), and 10 μL of nuclease-free water. The thermal cycling conditions were as follows: initial denaturation at 98 ℃ for 2 minutes; 40 cycles of denaturation at 98 ℃ for 10 seconds, annealing at 55 ℃ for 15 seconds, and extension at 68 ℃ for 40 seconds. Amplification was performed using a SimpliAmp™ thermal cycler (Applied Biosystems).

The PCR products were resolved on a 2% agarose gel prepared in 1× Tris-Acetate-EDTA (TAE) buffer. The amplified products were electrophoresed alongside a 50 bp DNA ladder (FastGene 50 bp DNA Marker) at 100 V for 30 minutes. The gel was subsequently stained with 0.8% (v/v) ethidium bromide in TAE buffer. DNA bands were visualized under UV illumination using a gel documentation system to assess the quality and size of the amplified products.

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions to remove residual primers and nucleotides. The purified amplicons were then sequenced by the Sanger method (SeqStudio Genetic Analyzer; Thermo Fisher Scientific, USA) in both forward and reverse directions using the same primer set (LCO1490 and

HCO2198). The resulting nucleotide sequences were quality-checked and submitted to GenBank.

2.3.2. Phylogenetic analysis of sand fly species

Sanger sequencing was performed on purified PCR amplicons of the mitochondrial *COI* gene obtained from sand fly specimens. Chromatograms were analyzed and manually edited using BioEdit Sequence Alignment Editor (version 7.2.5) to ensure sequence quality. The resulting sequences were subjected to homology searches using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) to verify identity and rule out potential contaminants.

For species identification, the obtained sequences were compared against the NCBI GenBank and Barcode of Life Data System (BOLD) databases. Multiple sequence alignments were performed using ClustalW implemented in Molecular Evolutionary Genetics Analysis (MEGA) software (version 11). Phylogenetic trees were constructed using the maximum likelihood method based on the lowest Bayesian Information Criterion (BIC) scores, with 1 000 bootstrap replicates in MEGA software. Intraspecific genetic divergence among sand fly species was estimated using the Kimura 2-parameter (K2P) model in MEGA software.

2.4. Ethics approval

This study was approved by the Ethics Review Committee of the Faculty of Medicine, University of Kelaniya, Sri Lanka (Reference No. P/95/07/2023; approved on 13 February 2024). The study involved the collection and examination of sand fly specimens and did not involve human participants, human biological samples, or identifiable personal data. Therefore, written informed consent was not required. All study procedures were conducted in accordance with the ethical standards of the institutional review board and the principles of the 1964 Declaration of Helsinki and its later amendments, where applicable.

3. Results

3.1. Morphological review of sand flies

A total of 58 sand fly specimens were examined through morphological analysis, and representative samples from each species were utilized for molecular identification. All specimens were received as part of the referral-based entomological surveillance system operated by the Department of Parasitology, University of Kelaniya, Sri Lanka and were selected specifically on the basis of discrepancies in morphological features relative to the existing taxonomy for Sri Lankan phlebotomines. Based on morphological characterization, the collection comprised five known species representing two genera, *Phlebotomus* and *Sergentomyia*, along with three morphologically distinct species not previously recorded in Sri Lanka. The identified species included *P. argentipes* (n=21, 36.2%), *S. zeylanica* (n=21; 36.2%), *S. indica* (n=1; 1.7%), *S. bailyi* (n=4; 6.9%), and *S. babu insularis* (n=4; 6.9%). In addition to these known taxa, two species with distinct morphological characteristics were encountered. Together, the five known species accounted for 87.9% of all specimens examined, with *P. argentipes* and *S. zeylanica* jointly dominating the collection at equal proportions (36.2% each), consistent with their previously documented abundance across Sri Lankan districts[16]. The two morphologically anomalous species collectively accounted for the remaining 12.1% (n=7) of the examined material. In total, nine new *COI* sequences were generated and analyzed from specimens collected in three districts of the country.

3.2. Geographical record of species noted with different morphologies

Morphological characterization revealed that one of these unknown species belonged to *Idiophlebotomus* sp. (n=5, 8.6%), a genus not previously reported from Sri Lanka. These *Idiophlebotomus* sp. specimens were received from Gampaha district (7.279 164°N, 80.080 019°E; 7.221 161 6°N, 80.065 118 6°E and 7.231 812°N, 80.152 155°E) and from the Galle district in the Southern Province (6.269 509°N, 80.293 887°E). The other two unidentified specimens were assigned to the genus *Sergentomyia* but did not match any known species, indicating the presence of a potentially novel *Sergentomyia* species in the country. These unknown *Sergentomyia* specimens were received from the Anuradhapura district in the North Central Province (8.007 319°N, 80.517 389°E), the district with the highest reported cutaneous leishmaniasis burden in Sri Lanka, situated in the dry zone and characterized by scrub-dominated vegetation and peri-domestic settlement patterns. Both specimens were collected from peri-domestic environments adjacent to fragmented forest using CDC light traps. The two *Sergentomyia* specimens were collected from the same location, and genetic distance analysis based on the K2P model confirmed negligible intraspecific divergence, supporting their placement within a single species. The male and female specimens were formally designated as *S. nayanasis* sp. nov. and submitted to GenBank under accession numbers PX226518 and PX127887, respectively.

3.3. Taxonomic description of sand fly species (Diptera; Psychodidae)

3.3.1. Identification of Idiophlebotomus sp. male

Idiophlebotomus sp. can be distinguished from other sand fly genera by the presence of a cibarium bearing teeth and an unarmed pharynx (Supplementary Figure 1). The palps do not extend beyond antennal

segment 3 (A3). In males, the style bears 3 spines, and a pair of intra-abdominal rods parallel to the pump and genital filaments is present. This combination of characters separates *Idiophlebotomus* from other genera, such as *Phlebotomus* and *Sergentomyia*[25,26].

3.3.2. *Idiophlebotomus sp. male*

The antennal segment 3 (A3) is longer than A4 + A5 (the combined length of antennal segments 4 and 5). Cibarium contains numerous denticles, which are arranged in multiple distinct rows (Figure 2A). Palpal formula is 1, 4, 2, 5, 3. Style is elongated and slender, equipped with three pointed spines, and these spines are neither terminal nor subterminal and are spaced apart from each other (Figures 2B and 2C). Coxite is broad and relatively large, bearing numerous long setae, especially dense at the apical end (Figures 2B and 2C). The genital filament is thin, and the paramere is narrow and elongate, lacking any dorsal or ventral processes (Figure 2C). Aedeagus is darkly pigmented, with a slightly swollen tip (Figure 2D). Large and wide wings with a length/width ratio of 3.08 (Figure 2E). The pharynx is slender with fine scales (Figure 2F).

3.3.3. *Identification of Sergentomyia nayanasis sp. nov.*

The presence of recumbent hairs on abdominal tergites 2–6 observed in both sexes is characteristic of the genus *Sergentomyia* (Supplementary Figures 2 and 3). The cibarial teeth are arranged in a transverse row, with a distinct pigment patch and upwardly directed fore teeth. Antennal segment 3 (A3) is longer than A4 + A5. The pharynx is slender and weakly armed. In females, the spermatheca is a thin-walled capsule, sometimes with cross-striations, with a narrow duct joining a common duct. In males, the aedeagus is slender with a blunt tip, and the paramere is hooked with a ventral hairy tubercle. The style bears four spines and an accessory seta, and the genital filament shows distinct transverse striations. These morphological characters confirm the placement of the present specimens within the subgenus *Neophlebotomus* of the genus *Sergentomyia*.

S. nayanasis sp. nov. can be distinguished from other species of the subgenus *Neophlebotomus* by the combination of a well-developed cibarial armature comprising 8–12 robust, darkly pigmented teeth arranged in a single transverse row, and a pharynx with fine longitudinal striations lacking heavily sclerotized spines. In females, the spermatheca is smooth, with a relatively wide individual duct, cross striations, and minute projections at the apical end. In males, the style bears four spines arranged in a characteristic pattern of two terminal and two subterminal spines. The paramere is curved downward with a rounded apex, and the aedeagus is comparatively thick and finger-shaped. This unique combination of characters distinguishes *S. nayanasis* sp. nov. from other described *Neophlebotomus* species (Table 1).

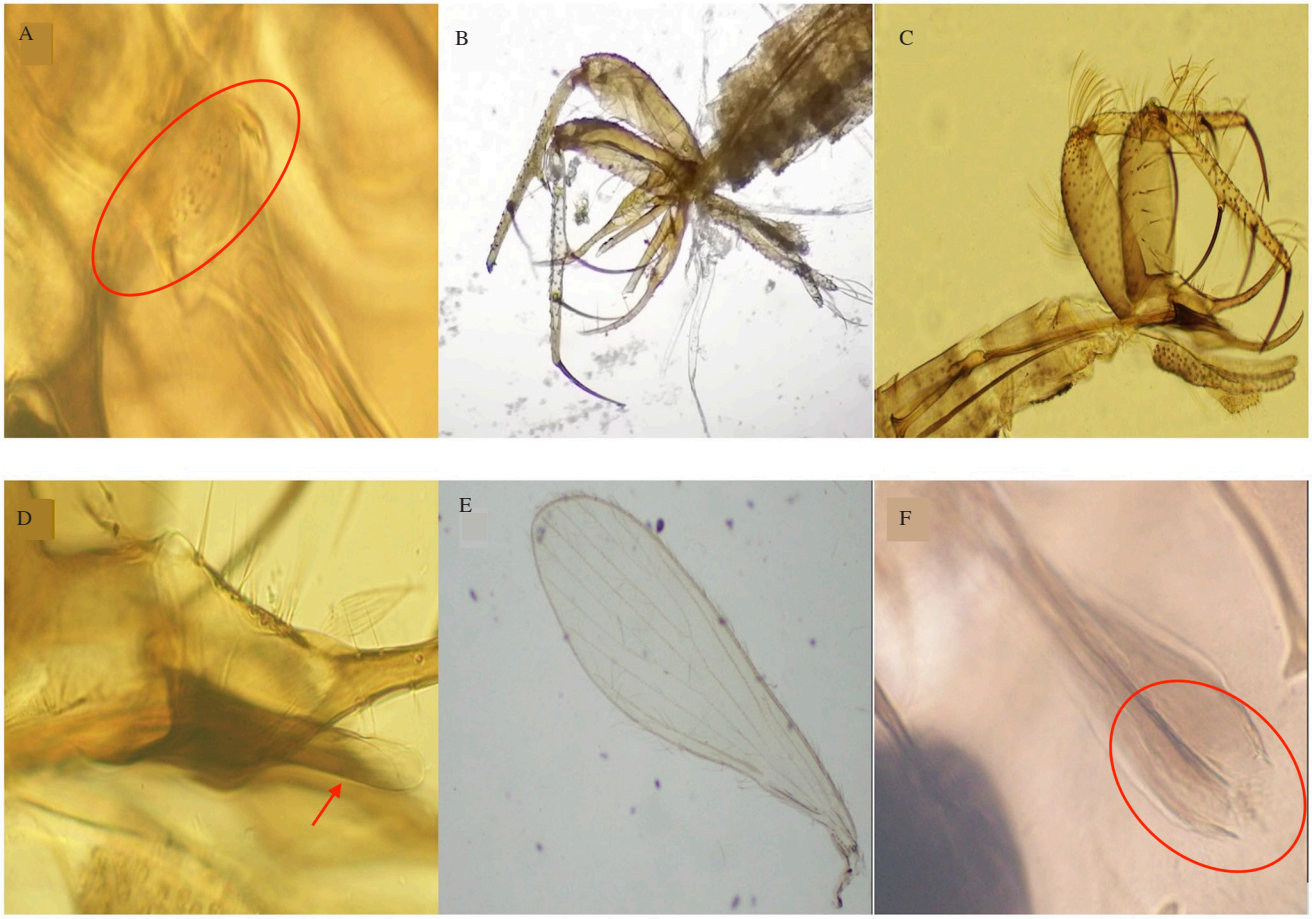

Figure 2. Morphological features of *Idiophlebotomus* sp. male (×100). A: Cibarium; B: Terminalia; C: Terminalia; D: Aedeagus; E: Wing; F: Pharynx. The circle in panel A highlights the cibarial teeth; the arrow in panel D highlights the aedeagus, and the circle in panel F highlights the pharyngeal armature.

Table 1. Morphometric measurements of adult male *Idiophlebotomus* sp., adult male *Sergentomyia nayanasis* sp. nov., and adult female *Sergentomyia nayanasis* sp. nov.

Measurement of the taxonomic feature (μm)	*Idiophlebotomus* sp., (*n*=2)	*Sergentomyia nayanasis* sp. nov. female (*n*=1)	*Sergentomyia nayanasis* sp. nov. male (*n*=1)
Head length	258.12-263.37	275.43	228.95
Head width	318.00-386.79	308.70	232.59
Antenna Ⅰ (A1)	487.49	Damaged	219.46
Antenna Ⅱ (A2)	160.42	Damaged	103.91
Antenna Ⅲ (A3)	149.68	Damaged	84.58
Palpomere length P1	15.02	Damaged	32.27
Palpomere length P2	43.18	Damaged	83.49
Palpomere length P3	71.86	Damaged	106.35
Palpomere length P4	35.50	Damaged	115.98
Palpomere length P5	61.96	Damaged	155.52
Wing length	1567.57-1580.24	Damaged	1218.61
Wing width	497.52-524.79	Damaged	302.54
Principal vein length			
Alpha	285.43	Damaged	176.56
Beta	213.29	Damaged	284.89
Gamma	124.85	Damaged	253.88
Delta	112.73	Damaged	51.99
Leg parameters			
Femur	782.03	Damaged	Damaged
Tibia	1066.02	Damaged	Damaged
Tarsomeres			Damaged
T1	652.35	Damaged	Damaged
T2	291.42	Damaged	Damaged
T3	189.40	Damaged	Damaged
T4	114.25	Damaged	Damaged
T5	103.24	Damaged	Damaged
Style	285.07-337.24	-	95.92
Coxite length	224.96-274.50	-	169.95
Coxite width	90.98-100.16	-	64.69
Individual surstyle	255.02-302.77	-	151.25
Paramere full length	230.64-240.97		141.91
Aedeagus	103.94-126.43	-	61.73
Genital filaments + pump	464.79-471.10		304.90
Genital pump	144.28-206.34	-	194.82
Length of spermatheca	-		-
Width of spermatheca	-	Spermatheca not visible	-
Number of segmentations in spermathecal	-	Spermatheca not visible	-

Notes: "–" indicates that the parameter is not applicable. Morphometric measurements were obtained from a single well-preserved slide-mounted specimen for the newly described *Sergentomyia* species. For *Idiophlebotomus* sp., measurements were taken from two slide-mounted specimens; however, antennal, palp, and leg antennal parameters could only be recorded from one specimen due to partial damage in the other. Due to degradation over time, some morphological structures (spermatheca) were no longer visible in certain specimens and therefore could not be measured. Despite these constraints, the morphometric data obtained, particularly the genital dimensions, wing length/width ratios, and antennal proportions, are sufficient to characterize both taxa and, together with the morphological descriptions above, distinguish them clearly from all previously recorded sand fly species in Sri Lanka[16]. The full set of measurements is provided to facilitate future comparative studies as additional specimens become available.

3.3.4. Sergentomyia nayanasis sp. nov. female

The cibarium exhibits a well-developed armature of 8-10 teeth with a series of robust, darkly pigmented cibarial teeth arranged in a transverse row along the posterior margin. These teeth are pointed and evenly spaced, forming a comb-like structure (Figure 3A). The pharyngeal armature appears as a series of longitudinally arranged ridges and striations, extending along the dorsal wall and converging toward the posterior end (Figure 3B). These striations are relatively fine and evenly spaced, without the presence of heavily sclerotized hooks or thick spines (Supplementary Figure 1). The spermathecae are smooth, with a wide individual duct. The apical end of the spermatheca with minute projections (Figure 3C).

3.3.5.Sergentomyia nayanasis sp. nov. male

Antennal segment 3 (A3) is longer than A4+A5. Cibarium with 10-12 teeth and arranged in one line (Figure 4A). The pharynx is lightly armed with scales (Figure 4B). The style bears four spines arranged in a typical pattern of two terminal and two subterminal spines. Outer hairs of the coxite are evenly spaced, and the paramere curves downward and has rounded ends. Aedeagus is thick, finger-shaped (Figure 4C).

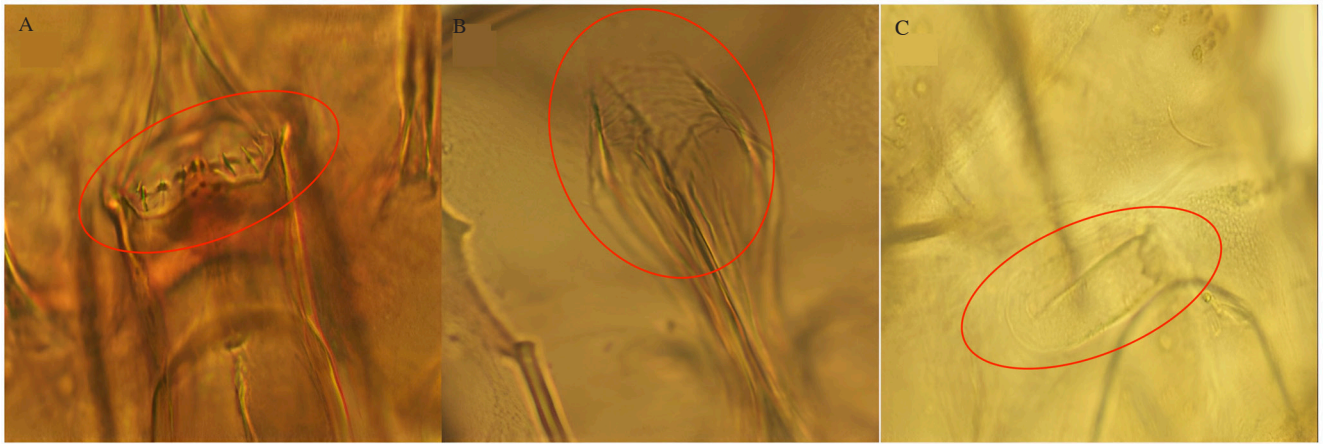

Figure 3. Morphological features of *Sergentomyia nayanasis* sp. nov. female (×100). A: Cibarium; B: Pharynx; C: Spermatheca. The circles in the panel A, B and C highlights the cibarial teeth, pharyngeal armature and spermatheca respectively.

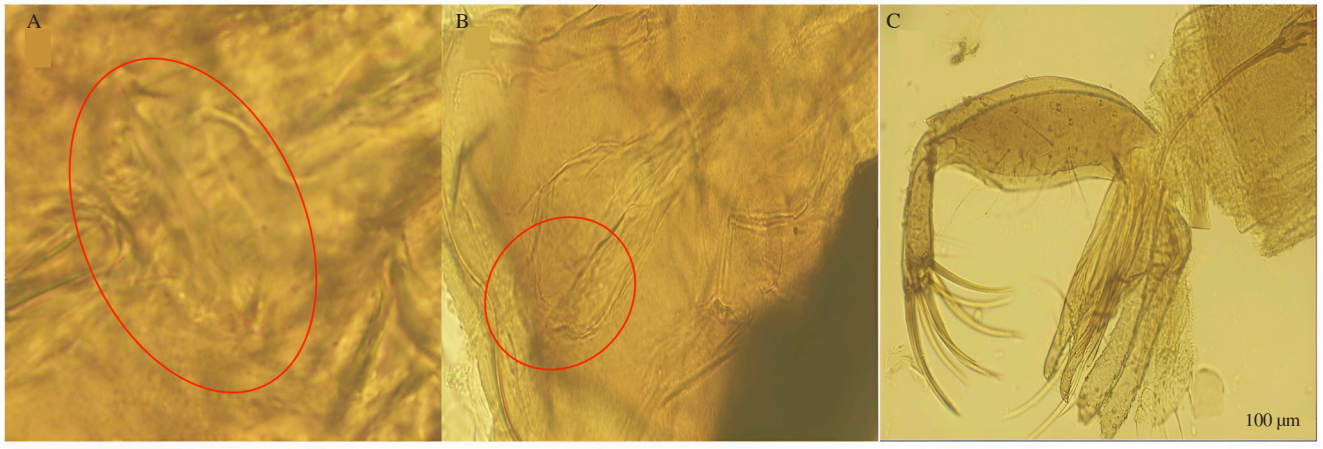

Figure 4. Morphological features of *Sergentomyia nayanasis* sp. nov. male (×100). A: Cibarium; B: Pharynx; C: Terminalia. The circles in the panel A, and B highlights the cibarial teeth, and pharyngeal armature respectively.

3.4. Variability

The morphometric characteristics indicated that the holotype female and paratype male were comparable, although they were collected from similar habitats on different dates. DNA barcode sequences of the examined specimens showed negligible intraspecific genetic divergence (K2P), supporting their placement within a single taxonomic entity. In contrast, the genetic divergence between these specimens and other congeners exceeded 5%, indicating clear separation from related species. Specifically, the intraspecific K2P distance between the holotype and paratype sequences was <1%, well within the threshold commonly accepted for conspecific individuals in arthropod *COI* barcoding studies[14]. The interspecific K2P divergence of approximately 5% relative to the closest congeners *S. khawi* and *S. iyengari* substantially exceeds the 2%–3% barcoding gap typically used to delimit species boundaries in phlebotomines and other Diptera[3], providing robust molecular corroboration for species-level recognition. The concordance between morphological distinctiveness and molecular divergence constitutes the strongest possible evidence for the validity of *S. nayanasis* sp. nov. as a new species under an integrative taxonomic framework.

3.5. Type materials

Voucher specimens, comprising both the holotype female and paratype male, were mounted separately on microscopic glass slides in Canada balsam and serially numbered with details on the place and date of collection, habitat type, and collector's name. The type specimens have been deposited in the Department of Parasitology, Faculty of Medicine, University of Kelaniya, Sri Lanka. Specimens of *S. nayanasis* sp. nov. processed for molecular analysis were submitted to GenBank under accession numbers PX226518 (male) and PX127887 (female).

3.6. Etymology

The new species *S. nayanasis* sp. nov. is named after Prof. Nayana Gunathilaka, Professor in Parasitology, Faculty of Medicine, University of Kelaniya, Sri Lanka, acknowledging his

supervision and contribution in the field of Medical Parasitology and Entomology in Sri Lanka especially to the sand fly taxonomy by developing the first morphological identification key for sand fly fauna in Sri Lanka.

3.7. Genetic confirmation and phylogenetic analysis

The phlebotomine sand fly isolates generated in this study were subjected to nucleotide sequence similarity analysis using the BLAST algorithm against the comprehensive NCBI GenBank nucleotide database to establish taxonomic identity through comparative sequence homology. In total, nine *COI* sequences were successfully generated: two from *Idiophlebotomus* sp. specimens and two from *S. nayanasis* sp. nov., with the remaining five sequences derived from morphologically confirmed known species used as internal controls to verify the sequencing pipeline. All sequences passed quality thresholds following manual editing in BioEdit and were deposited in GenBank prior to phylogenetic analysis. The maximum likelihood phylogenetic reconstruction based on mitochondrial *COI* gene sequences revealed well-resolved taxonomic groupings and variable levels of intraspecific genetic divergence (Figure 5).

The genus *Sergentomyia* formed several distinct monophyletic clades with high statistical support. *S. khawi* sequences (accessions PQ462431, PQ871070, PQ871074, PQ871072, PQ871069, PQ871077, PQ871073, PQ871079 and PQ871076) clustered within a well-supported clade (bootstrap value ≥84%), demonstrating genetic cohesion despite potential geographic or temporal sampling variation. *S. iyengari* sequences (PQ468936, PQ468935, PQ468934 and PQ468937) formed a sister clade with robust bootstrap support (≥92%), indicating clear species-level genetic differentiation from *S. khawi*. The sequences corresponding to *S. nayanasis* sp. nov. (PX226518 and PX127887) were positioned within the *Sergentomyia* clade and showed proximity to *S. khawi*. However, these sequences did not form a distinct, strongly supported monophyletic group and were distributed across adjacent branches with moderate bootstrap support.

The sequences assigned to *Idiophlebotomus* sp. (PV718003 and PV718009) did not cluster together and were placed in different regions of the phylogenetic tree. These sequences were associated

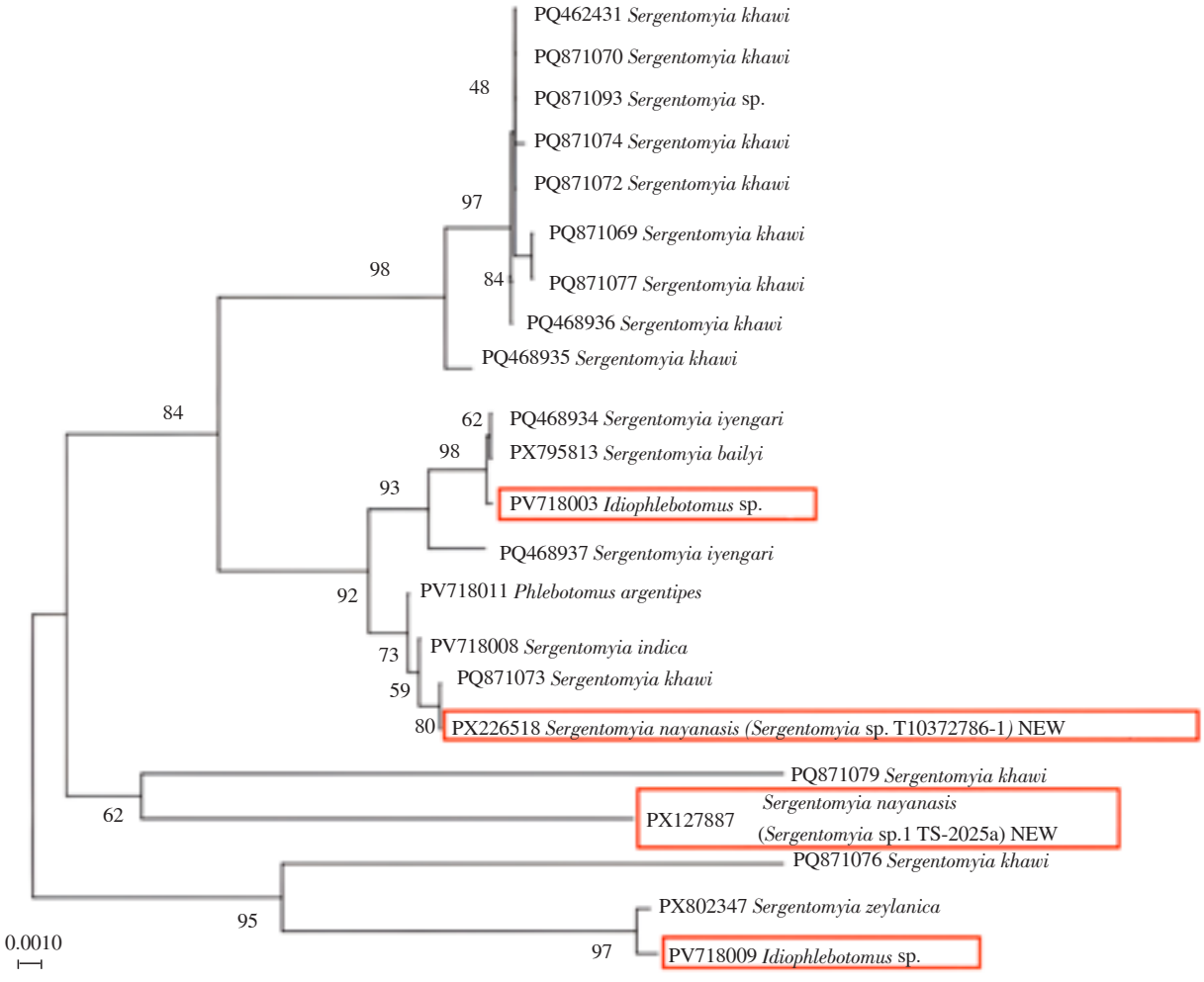

Figure 5. Phylogenetic tree of mitochondrial cytochrome c oxidase subunit I (*COI*) gene sequences for species *Idiophlebotomus* sp., and *Sergentomyia nayanasis* sp. nov., along with other sand fly species.

with clades containing species from multiple genera, and no well-supported grouping corresponding to *Idiophlebotomus* was observed. Bootstrap values ranged from 48% to 98%, with higher support values (≥80%) generally observed for terminal species-level clades, while deeper phylogenetic relationships showed more moderate support (59%-73%), likely reflecting rapid evolutionary diversification within this medically important dipteran subfamily. The *COI* gene region provided sufficient phylogenetic signal to resolve species-level relationships while maintaining sensitivity to detect cryptic diversity within morphologically similar taxa.

4. Discussion

According to the most recent checklist, Sri Lanka is known to harbour two sand fly genera, *Phlebotomus* and *Sergentomyia*. To the best of our knowledge, the present study confirms the occurrence of a third genus, *Idiophlebotomus*, in Sri Lanka, thereby expanding the known diversity of phlebotomine fauna in the region. In addition, this investigation reports a novel sand fly species from a district with a high prevalence of leishmaniasis, which is herein described as *S. nayanasis* sp. nov.

Previously, *Idiophlebotomus* was classified as a subgenus of the genus *Phlebotomus* and later elevated to the genus level[27]. According to the latest global checklist for sand flies by Galati *et al.*, there are 14 species under the genus *Idiophlebotomus* distributed across several regions, including Thailand [28], Nepal[25], Vietnam[29], Malaysia, Indonesia, Philippines, China, Cambodia, Australia, and India[30]. Despite its relatively wide distribution in the Oriental and Australasian regions, *Idiophlebotomus* has not previously been reported from Sri Lanka. Therefore, the present record represents the first report of this genus in the country, extending its known geographical range within South Asia.

The discovery of *Idiophlebotomus* sp. in Sri Lanka is biogeographically noteworthy and warrants careful consideration in light of the island's geological and ecological history. Sri Lanka shares close affinities with the Indian subcontinent, from which it was periodically separated and reconnected via land bridges during the Pleistocene glacial cycles, and its current isolation is maintained by the shallow Palk Strait and Gulf of Mannar[7]. The Western Ghats–Sri Lanka biodiversity hotspot is characterized by high levels of endemism across multiple taxonomic groups, shaped by both ancient vicariance and more recent dispersal events[8]. The detection of *Idiophlebotomus* in both the wet zone (Gampaha) and dry zone (Galle) of Sri Lanka suggests that the genus may be more broadly distributed across ecologically diverse habitats than previously recognized.

This ecological plasticity has been documented for other *Idiophlebotomus* species in Southeast Asian Island systems, where they have been collected from both humid forested environments and cave systems[27]. Notably, the closest geographically documented occurrence of *Idiophlebotomus* is in southern India, where members of this genus have been sporadically encountered in forested districts, although formal documentation remains limited[25]. The present finding, therefore, raises the intriguing possibility that *Idiophlebotomus* may have colonized Sri Lanka *via* dispersal across the historically intermittent land bridge connecting it to the Indian mainland, or alternatively that it represents a relic lineage that has persisted in the island's forested refugia. Molecular phylogeographic analyses incorporating specimens from India, Southeast Asia, and the newly reported Sri Lankan populations would be essential to test these hypotheses and to elucidate the historical dispersal routes of this genus within the Oriental region.

The identification of the Sri Lankan specimens as *Idiophlebotomus* sp. was primarily based on morphological characters, including the presence of cibarial teeth, an unarmed pharynx, short palps not exceeding antennal segment 3, and the presence of intra-abdominal rods associated with the male genitalia. These diagnostic features are consistent with the genus-level characteristics described in the previous literature and taxonomic descriptions. Molecular analysis of *COI* sequences of the examined specimens showed approximately 92% similarity to *Micropygomyia atroclavata* in BLAST analysis. In contrast, morphological examination clearly supports placement within the genus *Idiophlebotomus*, based on key diagnostic characters. The sequences assigned to *Idiophlebotomus* sp. (PV718003 and PV718009) did not cluster together and were distributed across different branches of the tree, forming no well-supported monophyletic group. This discrepancy between molecular similarity and morphology is likely due to the limited availability of reference sequences for *Idiophlebotomus* in public databases and the possibility of morphological and genetic divergence among the same taxa[30]. Therefore, the specimens are conservatively treated here as *Idiophlebotomus* sp., and further integrative taxonomic studies incorporating additional molecular markers and broader reference datasets are required to clarify their species status with a larger number of specimens.

Equally significant was the discovery of a novel species, *S. nayanasis* sp. nov., from the Anuradhapura district, a region with the highest incidence of cutaneous leishmaniasis in Sri Lanka[10]. The female of *S. nayanasis* sp. nov. showed some resemblance to *S. khawi*[22], particularly in the general morphology of the spermathecae. However, clear differences were observed. In *S. khawi*, the cibarium typically contains 10–12 hind teeth with two or three rows of fore teeth and a lightly developed pigment patch, whereas in *S. nayanasis* sp. nov., the cibarium possesses 8–10 robust, darkly pigmented teeth arranged in a single transverse row, forming a comb-like structure. Furthermore, the spermatheca of *S. khawi* is narrow with slight wrinkling and a distinct knob located within

a deep, narrow pit, while in *S. nayanasis* sp. nov., the spermatheca is smooth, with a relatively wide individual duct and the apical region bearing minute projections. The male of *S. nayanasis* sp. nov. is comparable to *S. iyengari*[31] in general morphology; however, several diagnostic differences were present. In *S. iyengari*, the cibarium bears 14–17 hind teeth, with fore teeth absent or variably arranged in one or two rows, and the pigment patch is variable in shape and development. In contrast, *S. nayanasis* sp. nov. exhibits 10–12 cibarial teeth arranged in a single row. Additionally, the male genitalia of *S. nayanasis* sp. nov. are characterized by a thick, finger-shaped aedeagus and a downwardly curved paramere with a rounded apex, which distinguishes it from *S. iyengari*.

The placement of *S. nayanasis* sp. nov. within the subgenus *Neophlebotomus* is consistent with recent trends toward the description of novel species in this taxonomically rich subgenus across South and Southeast Asia. The subgenus *Neophlebotomus* is diagnosed principally by the presence of a style with four spines in males and spermathecae with characteristic morphological features in females, characters clearly present in *S. nayanasis* sp. nov.[3]. Comparable integrative approaches combining morphological characters with *COI* barcoding have successfully delimited new *Sergentomyia* species in the Western Ghats of India, where *S. ashwanii* sp. nov. was recently described from a region geographically proximate to Sri Lanka[3]. This parallel suggests that the Western Ghats–Sri Lanka biodiversity hotspot may harbour considerably greater undescribed phlebotomine diversity than current records indicate, and that systematic sampling in forested habitats of the island, particularly in the central highlands and dry zone forests, is a research priority. The morphological distinctiveness of *S. nayanasis* sp. nov. further highlights the importance of robust pictorial keys and standardized morphometric protocols in facilitating accurate species discrimination, a challenge that has historically impeded progress in Sri Lankan sand fly taxonomy and was only partially addressed with the development of the first national pictorial key in 2020[16]. Moreover, while genetic distances exceeding 2%–3% in the *COI* barcode region are commonly used as thresholds for species-level discrimination in arthropods[14], the observed interspecific divergence of approximately 5% between *S. nayanasis* sp. nov. and its closest congeners provides compelling molecular evidence for its recognition as a distinct species. Similar thresholds of species-level *COI* divergence have been consistently applied in recent descriptions of new sand fly species from Laos[22] and Brazil[32], lending credibility to the barcoding approach adopted here.

Despite morphological convergence, mitochondrial *COI* sequence analysis revealed pronounced genetic differentiation, with the Sri Lankan specimens constituting a highly supported monophyletic lineage (bootstrap support=98%, indicating robust statistical confidence in clade authenticity). Pairwise genetic distance calculations demonstrated minimal intraspecific sequence divergence (<1%) within this clade, contrasting with substantial interspecific divergence (~5% K2P distance) from sister taxa, thereby satisfying standard DNA barcoding thresholds for species-level recognition and supporting the designation of a previously unrecognized taxonomic unit.

The maximum-likelihood phylogenetic reconstructions generated in this investigation substantiate the diagnostic efficacy of the mitochondrial *COI* barcode region for discriminating phlebotomine species and for phylogenetic inference. The topological placement of *Idiophlebotomus* sp. sequences (PV718003 and PV718009) within a well-supported clade alongside Neotropical congeners (bootstrap=97%) corroborates taxonomic identity through reciprocal monophyly, while the discrete phylogenetic position of the putative *S. nayanasis* lineage exemplifies the marker's sensitivity for cryptic species detection within morphologically conserved groups.

Notwithstanding the robust phylogenetic signal provided by *COI*, exclusive reliance on maternally inherited mitochondrial loci may fail to capture the full spectrum of evolutionary relationships, particularly in cases of mitochondrial introgression, incomplete lineage sorting, or male-mediated gene flow. Integration of biparentally inherited nuclear markers, including the ribosomal internal transcribed spacer 2 (ITS2) and *28S* rRNA gene regions as validated in previous Sri Lankan phlebotomine investigations[14,21] would provide enhanced phylogenetic resolution through multi-locus species delimitation approaches and facilitate detection of genealogical discordance indicative of reticulate evolutionary processes within morphologically cryptic species complexes.

The ecological niches occupied by the newly identified species suggest possible epidemiological significance. *Idiophlebotomus* sp. was associated with forested habitats rich in leaf litter and rocky substrates in the present study, which may provide breeding and resting sites. By contrast, *S. nayanasis* was captured in peri-domestic environments interspersed with forest fragments, indicating potential contact with humans and domestic animals. These ecological patterns warrant further investigation through targeted surveys, bloodmeal analysis, and parasite screening to assess vectorial roles. Although *Sergentomyia* species have historically been considered non-vectors of human leishmaniasis, growing evidence from Africa and Asia indicates that some species may harbour *Leishmania* DNA, suggesting overlooked epidemiological roles[2,33]. The presence of *S. nayanasis* in a leishmaniasis hotspot emphasizes the need for studies on its ecology, host-feeding preferences, and potential involvement in parasite transmission.

The epidemiological implications of the discovery of new sand fly species in leishmaniasis-endemic districts of Sri Lanka deserve particular attention. Cutaneous leishmaniasis in Sri Lanka is caused predominantly by *L. donovani*, which unusually manifests as cutaneous rather than visceral disease in this region, a clinical and

epidemiological anomaly that remains incompletely understood[10]. The Anuradhapura district, where *S. nayanasis* sp. nov. was discovered, consistently reports the highest incidence of cutaneous leishmaniasis in the country[10], and the peri-domestic capture of this species raises the possibility that it may play a role in transmission dynamics that have hitherto been overlooked. The detection of *Leishmania* DNA in *Sergentomyia* species in investigations from Thailand[33] lends further weight to the hypothesis that some *Sergentomyia* species may serve as bridge vectors or reservoir hosts, even if they are not the primary transmission agents. Indeed, *S. khawi*, the morphologically most similar species to *S. nayanasis* sp. nov., was recently shown to harbour *Leishmania orientalis* and *Trypanosoma* spp. DNA in endemic areas of southern Thailand, representing a significant finding in understanding the vector competence of non-classical sand fly vectors[33]. Given these phylogenetic affinities and ecological parallels, vector competence experiments involving *S. nayanasis* sp. nov. and Sri Lankan *L. donovani* isolates are strongly warranted. Such studies should ideally incorporate both xenodiagnosis assays and molecular screening of field-collected specimens to determine infection rates, parasite species and strains harboured, and the extent to which this new species participates in the transmission cycle in Anuradhapura and surrounding districts.

The present study also highlights the broader issue of underestimated sand fly diversity in South Asia and the critical role of integrative taxonomy in unmasking cryptic species. Sand fly fauna in Sri Lanka was long considered relatively depauperate by regional standards, but the findings of the present study, together with the earlier identification of cryptic species within the *S. bailyi* complex[17] and the revelation of cryptic diversity in *P. argentipes* as reported[13,14], collectively indicate that the true species richness of Sri Lankan phlebotomines is substantially greater than current checklists reflect. This systematic underestimation is not unique to Sri Lanka; comparable cryptic diversity has been revealed in Mexico[34] and Brazil[32], where the application of advanced molecular tools has routinely uncovered morphologically indistinguishable species with distinct ecological niches and potentially divergent vector competencies. The adoption of next-generation sequencing approaches, including metabarcoding of bulk trap collections and whole-genome sequencing of selected specimens, will likely further accelerate the pace of discovery. Furthermore, the inclusion of multiple genetic markers beyond *COI*, such as the ribosomal ITS2 and cytochrome *b* gene[14,21], will be indispensable for resolving genus-level discordances of the type encountered with *Idiophlebotomus* sp. In the present study, where morphological and molecular evidence pointed in divergent directions due to the scarcity of reference sequences in public databases. In this context, there is an urgent need for a coordinated, region-wide sand fly genomics initiative that encompasses India, Sri Lanka, and Southeast Asia, aimed at populating reference databases with sequences from morphologically verified voucher specimens. Such an initiative would transform the currently fragmented molecular reference landscape into a reliable resource for future surveillance, outbreak response, and systematic research.

This study has several limitations that should be considered when interpreting the findings. First, the molecular analysis relied primarily on a single mitochondrial marker (*COI*), which provided limited resolution for distinguishing closely related taxa and yielded poorly resolved phylogenetic relationships, including polytomies and inconsistent clustering among genera. In addition, the availability of reference sequences for certain taxa, particularly *Idiophlebotomus*, is limited in public databases such as GenBank and BOLD, restricting robust species-level identification and comparative analysis. Morphological identification, although essential for taxonomic placement, was also constrained by the condition of some specimens. Certain diagnostic characters, such as ascoid structures, were not clearly observable and therefore could not be included in the analysis. The study was conducted within a limited geographical area and may therefore not reflect the full diversity and distribution of sand fly species in Sri Lanka. Future studies incorporating broader sampling, additional molecular markers, and more comprehensive reference datasets are required to further resolve the taxonomy and phylogenetic relationships of these taxa. Particularly, night-landing and resting collections supplemented by CDC light traps across different agro-ecological zones and seasons would provide more representative data on the true abundance and habitat associations of the newly detected taxa. Passive surveillance through referral networks offers a cost-effective and epidemiologically targeted strategy for detecting unusual or novel sand fly species in the field, and should be systematically expanded and standardized as part of national vector surveillance programmes.

From a public health perspective, the discoveries reported here reinforce the imperative of integrating vector biodiversity data into national leishmaniasis control frameworks. The WHO's roadmap for neglected tropical diseases 2021–2030 calls for intensified surveillance of vectors and reservoirs as a cornerstone of disease elimination strategies. In Sri Lanka, where leishmaniasis incidence has been rising steadily over the past two decades[10], the identification of previously unknown sand fly species in high-burden districts represents both a scientific milestone and a public health signal that demands operational response. Specifically, standard entomological indices, such as sand fly density, species composition, and seasonal abundance, in the Anuradhapura and Gampaha districts should be revisited using the expanded species list provided by this study. Bloodmeal analysis using molecular techniques (*e.g.*, cytochrome b barcoding of host DNA in engorged females) would clarify the host-feeding patterns of *S. nayanasis* sp. nov. and *Idiophlebotomus* sp., providing critical information on their anthropophilic or zoophilic tendencies and thereby their epidemiological relevance. Additionally, landscape-level analyses

using geographic information systems (GIS) and remote-sensing data linking sand fly occurrence records with land-use change, deforestation, and human settlement patterns would enable predictive modelling of transmission risk in areas where newly detected species are present. Such a One Health approach, integrating entomology, parasitology, human and animal health, and ecological data, represents the most robust framework for evaluating and managing the evolving leishmaniasis burden in Sri Lanka.

In conclusion, this study highlights the hidden diversity of Sri Lankan sand flies, emphasizing the importance of integrative taxonomy. The new record of *Idiophlebotomus* sp. and the description of *S. nayanasis* sp. nov. not only expand the known phlebotomine fauna but also raise important questions regarding species distributions, evolutionary histories, and epidemiological relevance. Further surveillance incorporating morphological, molecular, and ecological approaches is critical for refining sand fly systematics and for informing vector control strategies in leishmaniasis-endemic regions of Sri Lanka.

Conflict of interest statement

The authors declare that they have no competing interests.

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Authors' contributions

YK contributed to the study design, literature review, data collection, data analysis, data interpretation, and preparation of the original manuscript. NG contributed to the study conceptualization and design, supervised the research, and participated in manuscript preparation, review, and editing. DA contributed to supervision, manuscript preparation, review, and editing. CS contributed to data collection, methodology development, visualization, and manuscript drafting. NS contributed to data analysis, data interpretation, and manuscript drafting. NM contributed to supervision, funding acquisition, and critical review of the manuscript. All authors read and approved the final version of the manuscript.

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