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On the systematics of *Phlebotomus betisi* and two new related species from Laos with proposal of the new subgenus *Lewisius*

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Abstract – *Phlebotomus betisi* was described from Malaysia and classified after its description in the subgenus *Larroussius*. It was the only species to have a pharyngeal armature composed of dot-like teeth and an annealed spermatheca whose head is carried by a neck in females. Males were characterized by having a style bearing five spines and a simple paramere. The study of sandflies originating from a cave in Laos enabled us to discover and describe two sympatric species close to *Ph. betisi* Lewis & Wharton, 1963 and new for Science: *Ph. breyi* Vongphayloth & Depaquit n. sp., and *Ph. sinxayarami* Vongphayloth & Depaquit n. sp. They were characterized morphologically, morphometrically, geomorphometrically, molecularly, and proteomically (MALDI-TOF). All approaches converged to validate the individualization of these species whose morphological differential characters lay in the two genders by the observation of the interocular suture and by the length of the last two segments of the maxillary palps. In males, the length of the genital filaments discriminates these species. Females are distinguished by the length of the ducts of the spermatheca as well as by the narrow or enlarged shape of the neck bearing their head. Lastly, the particular position of the spines of the genostyle coupled with molecular phylogeny led us to remove these three species from the subgenus *Larroussius* Nizulescu, 1931 and to classify them in a new subgenus: *Lewisius* Depaquit & Vongphayloth n. subg.

Key words: Phlebotomine sandflies, Laos, Phlebotomus, New subgenus, New species, Systematics.

Résumé – Sur la systématique de Phlebotomus betisi et de deux nouvelles espèces apparentées du Laos avec proposition du nouveau sous-genre Lewisius. Phlebotomus betisi a été décrit de Malaisie et fut classé après sa description dans le sous-genre Larroussius. C'était la seule espèce à posséder chez la femelle une armature pharyngienne composée de dents en forme de points et à avoir une spermathèque annelée dont la tête est portée par un cou. Les mâles se caractérisaient par un style porteur de cinq épines et par un paramère simple. L'étude de Phlébotomes originaires d'une grotte du Laos nous a permis de découvrir et de décrire deux espèces sympatriques proches de Ph. betisi Lewis & Wharton, 1963 et nouvelles pour la Science : Ph. breyi Vongphayloth & Depaquit n. sp., et Ph. sinxayarami Vongphayloth & Depaquit n. sp. Elles ont été caractérisées morphologiquement, morphométriquement, géomorphométriquement, moléculairement et protéomiquement (MALDI-TOF). Toutes ces approches convergent pour valider l'individualisation de chacune de ces espèces dont les caractères morphologiques différentiels reposent dans les deux sexes par l'observation de la suture interoculaire et par la longueur des deux derniers segments des palpes maxillaires. Chez les mâles, la longueur des filaments génitaux discrimine ces espèces. Les femelles sont distinguées par la longueur des conduits des spermathèques ainsi que par la forme étroite ou élargie du cou portant la tête de ces spermathèques. Enfin, la position particulière des épines sur le gonostyle couplée à une phylogénie moléculaire nous amène à extraire ces trois espèces du sous genre Larroussius Nitzulescu, 1931 pour les classer dans un nouveau sous-genre : Lewisius Depaquit & Vongphayloth n. subg.

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1 Introduction

Phlebotomine sandflies represent a tiny blood sucking insect group which has been proven to be the vector of several human pathogens for decades [2, 3]. In the Old World, sandflies belong to eight genera including *Phlebotomus* Rondani & Berté, 1840; *Sergentomyia* França & Parrot, 1919; *Spelaeophlebotomus* Theodor, 1948; *Spelaeomyia* Theodor, 1948; *Idiophlebotomus* Quate & Fairchild, 1961; *Parvidens* Theodor & Mesghali, 1964; *Grassomyia* Theodor, 1958 and *Chinius* Leng, 1987 [43].

Genus *Phlebotomus* is currently divided into 14 subgenera including *Phlebotomus* Rondani & Berté, 1840; *Adlerius* Nitzulescu, 1931; *Larroussius* Nitzulescu, 1931; *Anaphlebotomus* Theodor, 1948; *Australophlebotomus* Theodor, 1948; *Euphlebotomus* Theodor, 1948; *Paraphlebotomus* Theodor, 1948; *Synphlebotomus* Theodor, 1948; *Kasaulius* Lewis, 1982; *Abonnencius* Morillas Márquez, Castillo Remiro & Ubeda Ontiveros, 1984; *Transphlebotomus* Artemiev & Neronov, 1984; *Legeromyia* Rahola, Depaquit & Paupy, 2013; *Madaphlebotomus* Depaquit, Léger & Randrianambinintsoa, 2015 and *Artemievus* Depaquit, 2022 [9, 12, 14, 27, 38, 43, 46, 47].

The subgenus *Larroussius* was created by Nitzulescu in 1931 [30] using *Ph. major* Annandale, 1910 [4] as the type species. Based on morphological characters, the *Larroussius* subgenus was originally described as follows: (i) cibarium without armature, (ii) spermathecae segmented with a long (variable) terminal process (neck) carrying the terminal knob, and (iii) pharynx similar to that of *Ph. major*; Theodor, 1948 [46] proposed: (i) short style with five long spines with two terminal spines and three spines near the middle of segment, (ii) simple parameres with a club-shaped apex, (iii) long parameral sheath (aedeagus) of variable shape, (iv) pharynx with armature of numerous small point-like teeth, and (v) spermathecae segmented with a long terminal process.

In Southeast Asia (SE-Asia), the only known species classified in the subgenus Larroussius is Ph. betisi Lewis & Wharton, 1963 [29]. This species was described from eight females caught in a cave in Betis, Gua Musang (Malaysia). These authors noted that Ph. betisi spermathecae look like those of Ph. major (i.e., close to those of Larroussius) but noticed that the bead-like segments and the narrowness of the process (meaning the terminal knob (head) carried by a neck) are unusual and that the systematic position of Ph. betisi could be clarified by the discovery of a male specimen. In 1978, Lewis [28] classified Ph. betisi in the subgenus Larroussius, a position adopted in papers published later by Lewis, 1982 [27]; Artemiev & Neronov, 1984 [9]; and by Seccombe et al., 1993 [43]. In 2008, the male of Ph. betisi was described by Khadri et al. [25] from specimens caught in Kota Gelanggi cave of Pahang, 20 km away from the type-locality. In this description, the authors indicated that Ph. betisi male specimens could be classified in the subgenus Larroussius by the morphology of the genitalia (meaning a gonostyle with 5 spines, a simple paramere without club-shaped apex or without a ventral tubercle, and the lack of basal process on the gonocoxite). The position of spines on the style, however, was different from that of other Larroussius specimens.

In the present paper, we describe two species new to science from Laos, closely related to *Ph. betisi*, and propose to create a new subgenus to include these three species.

2 Methods

2.1 Sandfly collection

Sandflies were collected between May 11 and May 17, 2019 from karstic limestone near the entrance of Pha Nok Kok cave in Feung district, Vientiane province, Laos (locality: $18^{\circ}30'$ N, $101^{\circ}59'$ E, Fig. 1). Sandflies were collected overnight (from 5:00 pm to 6:00 am) using standard CDC light traps (John W. Hock Company, Gainesville, FL, USA). Sandfly samples were then sorted and stored in 95% ethanol. An aliquot was stored dry in silica gel at -20 °C then transferred to the laboratory for further analysis.

2.2 Sample processing and morphological analysis

Specimens stored in 95% ethanol were mounted:

- in toto for morphological analysis: head, thorax, wings and genitalia were cut-off in a drop of ethanol. Soft tissues were lysed in a bath of 10% KOH then bleached in Marc-André solution, and mounted between microscope slide and cover slide in Euparal[®] for species identification after dehydration in successive alcoholic baths.
- Partially for molecular studies: head, wings and genitalia were cut-off and mounted directly in Euparal[®] as described above. Thorax and abdomen were transferred to an Eppendorf 1.5 mL tube with the same labelling as the slide and stored at -20 °C until analysis. All engorged females were processed according to this protocol.

For MALDI-TOF, dry specimens stored at -20 °C were prepared as follows: head, wings and genitalia were cut-off and mounted directly in either Macroinvertebrate Mounting Medium (Polysciences, Inc. Warrington, PA, USA) or in Euparal[®] as described above. Thorax and abdomen were transferred separately to two Eppendorf 1.5 mL tubes with the same labelling as that of the slide and stored at -20 °C until MALDI-TOF (using the thorax) and molecular analysis (using the abdomen).

The mounting slides were observed on an Olympus BX50 microscope coupled with a DP 26 Olympus camera. Measurements and counting of several characters were performed by using Stream Essentials software (Olympus, Tokyo, Japan), as previously explained [11]. Drawings were made using the *camera lucida* installed on the microscope.

2.3 Molecular analysis

2.3.1 Cytochrome b gene (cyt b)

The abdomen of the specimen stored in a 1.5 mL vial was processed by adding 0.5 mL of $1 \times$ Phosphate Buffered Saline



Figure 1. A: the star indicates the location of Pha Nok Kok cave in Feung district, Vientiane province, Laos; B: Pha Nok Kok cave location in the hills (red arrow); C: Pha Nok Kok cave entrance where the sandflies were caught.

(PBS) and Lysing Matrix E zirconium beads (MP Biomedicals, Santa Ana, CA, USA) and homogenized for 10 min at a vibration frequency of 25/s in a TissueLyser II system (QIAGEN, Hilden, Germany). After grinding, beads and tissues were spun down by centrifugation for 5 min at 3000 rpm. To obtain total nucleic acid, 100 µL of each sample were extracted and purified using a NucleoSpin[®]8 extraction kit, following the manufacturer's protocol and using an elution volume of 100 µL. All polymerase chain reaction (PCR) amplifications were carried out in a 50 µL volume containing 5 µL of extracted DNA and 45 µL PCR Master Mix (Promega, Madison, WI, USA), containing 50 pmol of each primer targeting cyt b: C3B-PDR (5'-CAYATTCAACCWGAATGATA-3') and N1N-PDR (5'-GGTAYWTTGCCTCGAWTTCGWTATGA-3'), according to previously published conditions [17]. Sequencing reactions were performed using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Waltham, MA, USA). Sequence chromatograms from both strands were obtained on an automated sequence analyzer ABI3500XL (Applied Biosystems).

Phylogenetic analysis was based on aligned sequences. The maximum likelihood (ML) tree was constructed by MEGA 11 [45] using the substitution models selected by Model test [36] with an Akaike information criterion (AIC) of HKY85 [21]. We have included five specimens of *Ph. breyi* n. sp. and five specimens of *Ph. sinxayarami* n. sp. as well as two Malaysian specimens of the closely related *Ph. betisi* and 53 specimens representing 53 species of the genus *Phlebotomus*, as indicated in Table 1. *Idiophlebotomus longiforceps* was selected to serve as an outgroup.

2.3.2 Blood meal analysis

The abdomen of each engorged female was processed individually. Total DNA was extracted with a QIAamp DNA mini kit (QIAGEN GmbH), according to the manufacturer's recommendations. The extracted DNA was eluted in a final volume of 100 μ L of AE buffer. For each extraction run, we included a positive control.

To check the bloodmeal origin of engorged females, we amplified the prepronociceptin (PNOC) gene using PNOC-F (forward): 5'–GCATCCTTGAGTGTGAAGAGAA–3' and PNOC-R (reverse): 5'–TGCCTCATAAACTCACTGAACC–3' primers, according to the conditions described in the literature [20]. Amplicons were analyzed by electrophoresis in 1.5% agarose gel containing gelgreen. Direct sequencing in both directions was performed with the primers used for DNA amplification.

2.4 MALDI-TOF MS Analysis

Matrix Associated Laser Desorption-Ionization – Time Of Flight mass spectrometry (MALDI-TOF) was performed as already described [22]. Briefly, thoraxes, including legs of the specimens, were placed in formic acid (10 μ L; Sigma-Aldrich, Lyon, France) and manually ground with a Teflon pestle. Acetonitrile (10 μ L Sigma-Aldrich) was then added and after a brief centrifugation step (2 min at 10,000 rpm), 1 μ L of supernatant was spotted on a 96-well steel MALDI target plate (Bruker Daltonics, Champs-sur-Marne, France). Specimens were deposited in quadruplets. After complete drying,

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Table 1. Specimens used for the molecular study.

Genus	Subgenus	Species	Origin	GenBank accession number
Phlebotomus	Larroussius	Ph. longicuspis	Burkina Faso	AY700012
		Ph. perfiliewi	Algeria	KF680820
		Ph. galilaeus	Cyprus	KF680828
		Ph. transcaucasicus	Iran	KF680835
		Ph. ariasi	France	HM131112
		Ph. chadlii	Algeria	HM131080
		Ph_orientalis	Sudan	KU559573
		Ph perniciosus	Tunisia	MW305409
		Ph keshishiani	Afghanistan	HO204193
		Ph tobhi	Cyprus	OI 376918
		Ph neglectus	Greece	OI 376973
		Ph sprideus	Icrael	KC329644
		Ph langeroni	Spain	I T223550
	Lowisius suba nov	Ph hatisi	Malaysia	00784674
	Lewisius subg. nov.	Ph hatisi	Malaysia	00784675
		Ph. bravi n. sp. male	Laos	00784676
		Ph. brevi n. sp. male	Laos	00784677
		Ph. brevi n. sp. male	Laos	00784678
		Ph. breyi n. sp. famala	Laos	00784670
		Ph. brevi n. sp. female	Laos	00784690
		Ph. breyt II. sp. female	Laos	00784080
		Ph. sinxayarami n. sp. male	Laos	00784681
		Ph. sinxayarami n. sp. female	Laos	00784682
		Ph. sinxayarami n. sp. male	Laos	OQ784683
		Ph. sinxayarami n. sp. male	Laos	OQ784684
		<i>Ph. sinxayarami</i> n. sp. female	Laos	OQ784685
	Adlerius	Ph. comatus	Iran	JX885988
		Ph. longiductus	Iran	JX885993
		Ph. kabulensis	Iran	JX885994
		Ph. halepensis	Iran	JX885995
		Ph. brevis	Iran	JX885998
		Ph. arabicus	Israel	KC329634
		Ph. creticus	Greece	MT501636
		Ph. balcanicus	Iran	MT501639
		Ph. chinensis	China	HM747234
		Ph. turanicus	Afghanistan	HM803195
		Ph. simici	Greece	MT552618
	Transphlebotomus	Ph. mascittii	Slovenia	MG800324
		Ph. canaaniticus	Israel	KC329646
		Ph. simonahalepae	Romania	MZ647524
		Ph. killicki	Greece	OL376894
	Anaphlebotomus	Ph. stantoni	Vietnam	KM409498
		Ph. rodhaini	Senegal	KM409501
	Euphlebotomus	Ph. argentipes	India	KM409508
		Ph. kiangsuensis	Thailand	OQ784686
		Ph. mascomai	Thailand	OQ784687
		Ph. barguesae	Thailand	KM409509
	Madaphlebotomus	Ph. berentiensis	Madagascar	KM409502
		Ph. fontenillei	Madagascar	KM409504
		Ph. vaomalalae	Madagascar	JX512360
		Ph. vincenti	Madagascar	KM409505
		Ph. artemievi	Madagascar	MN346688
		Ph. fertei	Madagascar	KM409506
	Phlebotomus	Ph. bergeroti	Algeria	KJ480973
		Ph. papatasi	Jordan	KY990733
		Ph. duboscqi	Cameroon	MH577174
	Paraphlebotomus	Ph. sergenti	Iran	DQ840405
	*	Ph. caucasicus	Iran	EF017364
		Ph. riouxi	Tunisia	EU935827
		Ph. chabaudi	Tunisia	EU935814
		Ph. jacusieli	Israel	KC329638
		Ph. saevus	Israel	KC329640
		Ph. similis	Greece	OL376910
		Ph. kazeruni	Israel	KC329635
	Artemievus	Ph. alexandri	Algeria	KJ480981
	Synphlebotomus	Ph. saltiae	Israel	KF483677
Idiophlebotomus	·····	Id. longiforceps	Thailand	KT878756

1 μ L of HCCA matrix solution (Bruker Daltonics) was added and the plates dried at room temperature. Spectra were then acquired using a Bruker Microflex LT MALDI–TOF spectrometer. For each well, spectra acquisition was repeated at least eight times. The Bacterial Test Standard provided by Bruker Daltonics was used for instrument calibration. Lastly, spectra were visually checked using FlexAnalysis v3.4 and imported in Biotyper Compass Explorer v4.1.100 for analysis.

The Bruker "MALDI Biotyper Preprocessing Standard Method" was used for the creation of main spectra profiles (MSP). High-quality spectra were selected for incrementing the local *Ph. Larroussius* database (at least 10 good quality spectra by MSP). Hierarchical cluster analysis (HCA) was performed using both the Euclidean and correlation method and the Ward algorithm for clustering with the MSP dendrogram tool of Compass Explorer.

Primary Component Analysis (PCA) was performed using custom R scripts based on the MALDIQuant, MALDIrppa and FactoMineR packages [19, 26, 31].

2.5 Geometric morphometric analysis

Geometric morphometric analysis [37] was carried out on 50 female sandfly wings (*Ph. sinxayarami* n = 39 and *Ph. breyi* n = 11) and 19 male sandfly wings (*Ph. sinxayarami* n = 10and *Ph. breyi* n = 9). Briefly, fuchsin-stained wings were photographed under ×100 magnification. Work files were then built with TPS Util[©] version 1.76, and 16 landmarks (LM) were digitized with TPSDig[©] version 1.40 [42]. Coordinates of scaled 16 landmarks were imported in R software Version 1.4.1103 [41] and processed with the geomorph package [1, 10]. Coordinates were aligned by performing a procrustes superimposition and centroid sizes (CS) were computed. Disparity between CS from the different novel species and between sexes was tested by means of a Wilcoxon test with p-values < 0.05 considered significant. Main shape LM disposition from male (n = 8) and female (n = 3) Ph. betisi was computed to serve as a reference for comparison with Ph. breyi and Ph. sinxayarami. Deformation grids were plotted to identify the main differences between LM positions. Principal Component Analysis (PCA) was performed on Ph. brevi and Ph. sinxayarami to assess whether morphologic differences could be used to separate these species. Plots were generated with R packages geomorph, factominer, factoextra and ggplot2 [26, 50].

3 Results

3.1 Molecular analysis

3.1.1 Cytochrome b gene

The 405 bp database included 223 variable sites and 182 informative sites for parsimony. A maximum likelihood tree based on this database is shown in Figure 2. The estimation of evolutionary divergence over sequence pairs between and within subgenera is provided in Table 2 and between and within

the species *Ph. betisi*, *Ph. breyi* n. sp. and *Ph. sinxayarami* n. sp. in Table 3.

The maximum likelihood phylogenetic tree of cyt b showed that Ph. betisi from Malaysia, Ph. breyi and Ph. sinxayarami are grouped together. The subgenus Madaphlebotomus, although appearing as paraphyletic, is a sister-group of these species and is separated from subgenus Larroussius with strong maximum statistical support (bootstrap = 100%) (Fig. 2). This result confirms our observations on morphologic characters of Lewisius n. subg. (as described below). Phylogenetic analysis also supports our morphologic association of male and female. Phlebotomus breyi of both sexes have long palps and incomplete interocular sutures. Females have long spermathecal ducts and males have long aedeagal ducts. Both sexes of Ph. sinxayarami have shorter palps, especially p4, and complete interocular sutures. Females have shorter spermathecal ducts and males have shorter aedeagal ducts than those of Ph. breyi (see details in description below).

The genetic distances between and within subgenera and species are listed in Table 2 and Table 3, respectively and highlight important differences.

3.1.2 Bloodmeal analysis

The blood meal of one engorged *Ph. sinxayarami* n. sp. female was successfully identified. The sequence homology was >99% with several sequences (including GenBank accession number XM_044939717.2) of water buffalo (*Bubalus bubalis*) that were observed around the entrance of Pha Nok Kok cave.

3.2 Wing morphometrics

Centroid sizes of *Ph. breyi* and *Ph. sinxayarami* are represented in Figure S1. The Wilcoxon test performed on CS showed no differences between the size of females from *Ph. breyi* and *Ph. sinxayarami* (*p*-value = 0.1622). In contrast, a significant difference between the two male populations was found (*p*-value = 2.646×10^{-5}). Procrustes analysis on mean shapes showed that the differences between *Ph. breyi* and *Ph. sinxayarami* rely mostly on deviations of LM Nos. 1 and 12 (Fig. S2). Plots of PCA individuals were able to discriminate between the two species, although both plots represented a small proportion of the total variance (56.7% and 47.3% for males and females, respectively) (Fig. 3).

3.3 MALDI-TOF

High-quality spectra were obtained from all 12 specimens of *Lewisius* n. subg. All spectra returned lower log-score values than threshold (1.7) when matched against our existing *Ph. Larroussius* group MSP database (data not shown). Hierarchical clustering (Fig. 4) allows one to (i) group all spectra (Fig. S3) from *Ph. breyi* n. sp. and *Ph. sinxayarami* n. sp. into a cluster distinct from spectra of specimens of the *Larroussius* group, and (ii) reliably discriminate between spectra from the two new species. The PCA analysis also enabled us to separate







Figure 2. Maximum likelihood phylogenetic tree of partial cyt b gene of the species within *Lewisius* n. subg. and *Larroussius* subgenus. Numbers on branches estimated by 100 replication bootstrap support. Species within *Lewisius* are separated from those within *Larroussius* with a bootstrap value of 100%.

	Lewisius	Larroussius	A dlerius	Transphlebotomus 🛛	4naphlebotomus	Euphlebotomus	Madaphlebotomus	Paraphlebotomus	Artemievus	Synphlebotomus	Phlebotomus Ia	liophlebotomus
Lewisius subg. nov.	0.102											
Larroussius	0.185	0.093										
Adlerius	0.180	0.133	0.074									
Transphlebotomus	0.194	0.147	0.127	0.122								
Anaphlebotomus	0.222	0.196	0.187	0.200	0.179							
Euphlebotomus	0.219	0.190	0.186	0.196	0.184	0.146						
Madaphlebotomus	0.175	0.173	0.177	0.196	0.215	0.211	0.128					
Paraphlebotomus	0.203	0.181	0.162	0.199	0.222	0.219	0.213	0.139				
Artemievus	0.231	0.204	0.184	0.204	0.246	0.227	0.233	0.205	n/a			
Synphlebotomus	0.176	0.171	0.146	0.186	0.206	0.197	0.202	0.164	0.157	n/a		
Phlebotomus	0.201	0.181	0.158	0.174	0.222	0.216	0.210	0.192	0.188	0.142	0.126	
Idiophlebotomus	0.224	0.217	0.222	0.237	0.242	0.215	0.230	0.257	0.258	0.230	0.250	n/a

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spectra from *Ph. breyi* from those of *Ph. sinxayarami* using only PCA 1 (Fig. 5), despite the low proportion of the variance explained by this component (5.1%).

3.4 Description of new taxa

Consensual terminology has been used in this description [18].

In our opinion, the species described below cannot be included in the subgenus *Larroussius* (see Discussion).

3.4.1 Description of *Lewisius* Depaquit & Vongphayloth n. subg.

urn:lsid:zoobank.org:act:959CB10D-E575-4313-AC52-9D192608CDFB

Genus: Phlebotomus Rondani & Berté.

Type species: Phlebotomus betisi Lewis & Wharton, 1963. *Lewisius* n. subg. is defined by (i) pharynx with armature of numerous small point-like teeth similar to those of *Larroussius*, (ii) gonostyle exhibiting five long spines out of which two are terminal, the upper external implanted subapically, the lower external implanted in the apical third and the inner one in its middle, (iii) simple parameres, (iv) conical parameral sheath, regularly tapering, and (v) segmented spermathecae, bead-like rings with a long terminal process.

Etymology: Lewisius refers to David J Lewis, Medical Entomologist at the Natural History Museum of London, who was a pioneer in the study of Phlebotomine sandflies of South-Eastern Asia and also described *Phlebotomus betisi*, the type-species of this new subgenus.

3.4.2 Description of *Phlebotomus breyi* Vongphayloth & Depaquit n. sp. (Figs. 6 and 7)

urn:lsid:zoobank.org:act:4BC1E78D-8CA0-416A-9214-99A4FBA6BC39

Genus: Phlebotomus Rondani & Berté, in Rondani 1840. *Subgenus: Lewisius* Depaquit & Vongphayloth n. subg. *Type locality:* Pha Nok Kok cave (18°30' N, 101°59' E),

Feung district, Vientiane province, Laos.

Type specimens: Holotype male (voucher LAOS 384-16) deposited in the Laboratory of Entomology of the Muséum National d'Histoire Naturelle de Paris (identification number MNHN-ED-ED11196). Two female and two male paratypes deposited at the Laboratory of Entomology of the Muséum National d'Histoire Naturelle de Paris (identification numbers MNHN-ED-ED11197, MNHN-ED-ED11198, MNHN-ED-ED11199, MNHN-ED-ED11200).

One male and one female paratype deposited at the Natural History Museum, London, UK (identification numbers NHMUK014908972 and NHMUK014908973).

One female and one male paratype deposited at the Laboratory of Medical Entomology, IPL.

Etymology: Epithet *breyi* refers to our Entomologist colleague Paul Brey, who created the Institut Pasteur du Laos (IPL) and the laboratory of Medical Entomology/Vector-borne diseases within the IPL.

Note: The authors of the new taxa are different from the authors of this paper: Article 50.1 and Recommendation 50A of the International Code of Zoological Nomenclature [24].

Table 3. Number of base differences per site from averaging over all sequence pairs between Lewisius subg. nov. species (thin style) and within Lewisius subg. nov. species (bold style) calculated using a p-distance model on the cyt b dataset.

	Ph. betisi Ph. breyi n. sp. P	Ph. sinxayarami 1		
h. betisi h. breyi n. sp. h. sinxayarami n. sp.	0 0.145 0.140 0.152	0.008		
<u>h. sinxayarami n. sp.</u>		0.008		
-8	-10 -5 0 5 10 -10 -5 0 5 10			

Figure 3. Morphometric analysis. Principal Component Analysis of wings landmarks of Phlebotomus (Lewisius) breyi n. sp. and Phlebotomus (Lew.) sinxayarami n. sp. for females (A, on the left) and males (B, on the right). Biplot of the first two principal components (PC1 and PC2).

3.4.3 Description of the male Ph. brevi n. sp. holotype (specimen LAO 384-16) (Fig. 6)

3.4.3.1 Head (Fig. 6A)

Occiput with several lines forming a thick stripe in the posterior occiput and a narrow line along the superior part of the eyes.

Clypeus 155 µm long exhibiting 35 setae. Anterior limit difficult to observe.

Eyes: 195 µm long, 113 µm wide, with about 80 facets. Incomplete interocular sutures.

Flagellomeres f1 (=AIII) = 289 μ m, f2 (=AIV) = 133 μ m, f3 (=AV) = 129 μ m.

Flagellomere 1 longer than $f^2 + f^3$.

Presence of two short ascoids never reaching the next articulation from f1 to f8, 1 from f9 and 1 atrophied, 1 from F10 and f11, and no ascoid on f12-f14.

Ascoidal formula: 2/f1-f8, 1 + 1 atrophied/f9, 1/ f10-f11, 0/ f12-f14.

Ascoid/f2 length ratio: 0.30

One distal papilla on f1 and f2 (Fig. 6B). Lack of papilla from f3 to f11. One papilla on f11, Four papillae on f12, four on f13, and four on f14.

No simple seta from f1 to f7. Presence of one or several simple setae from f8 to f14.

Palps (Fig. 6D): $p1 = 45 \mu m$, $p2 = 167 \mu m$, $p3 = 183 \mu m$, $p4 = 150 \ \mu m, \ p5 = 404 \ \mu m.$

Palpal formula: 1, 4, 2, 3, 5

Presence of a dozen of club-like Newstead's sensilla on p3. No Newstead's sensillum on other palpal segments (Fig. 6E).

Presence of one simple seta on distal p3; six on p4; more than 25 on p5. No simple seta on p1 and p2.

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Figure 4. MALDI-TOF analysis. Main Spectra Profile Dendrogram using correlation distance measures and Ward algorithm for *Lewisius* n. subg. and reference spectra of our in-house database [22] of the *Larroussius* subgenus.



Figure 5. MALDI-TOF analysis. Primary Component Analysis of the spectra of *Phlebotomus* (*Lewisius*) *breyi* n. sp. and *Phlebotomus* (*Lewisius*) *sinxayarami* n. sp. Biplot of the first two principal

Labrum 244 μ m long. Limit between the labrum and the clypeus difficult to observe.

Labial furca closed (Fig. 6C).

components (PC1 and PC2).

Cibarium armed with many tiny teeth pointed backwards (Fig. 6F).

Little pharyngeal teeth, commonly dot-like, sometimes pointed, and oriented backwards. All teeth are arranged along parallel curved lines.

Absence of sclerotized area.

3.4.3.2 Cervix

Two cervical sensilla. Two ventro-cervical sensilla.

3.4.3.3 Thorax

560 µm long.

Light brown sclerites.

Mesonotum: post-alar setae non-observed.

Pleurae: five proepimeral setae; absence of the upper and lower anepisternal, anepimeral, metaepisternal and metaepimeral setae; presence of fine setae on the anterior region of the katepisternum, and absence of the suture between metaepisternum and katepimeron. Metafurca mounted in lateral view on all specimens.

Wings (Fig. 6G): length = 1896 μ m; width = 595 μ m. $r5 = 1265 \ \mu$ m, $\alpha \ (r2) = 430 \ \mu$ m, $\beta \ (r2 + 3) = 176 \ \mu$ m, $\delta = 108 \ \mu$ m, $\gamma \ (r2 + 3 + 4) = 409 \ \mu$ m, $e \ (r3) = 581 \ \mu$ m, $\theta \ (r4) = 829 \ \mu$ m, $\pi = 0 \ \mu$ m. Width/ $\gamma = 1.45$.

Legs: Anterior leg: coxa = 309 μm ; femur = 757 μm , tibia = 970 μm , and tarsomeres ti = 647 μm , tii–tv = 719 μm .

Median leg: coxa = 343 $\mu m;$ femur = 742 $\mu m,$ tibia = 1117 $\mu m,$ and tarsomeres not observed.

Posterior leg: coxa = 330 μ m; femur = 786 μ m, tibia = 1378 μ m, tarsomeres ti = 798 μ m, tii-tv = 827 μ m.

3.4.3.4 Abdomen

Tergites ii–v: presence of randomly distributed setae. Tergites ii–vii: absence of tergal papillae.

3.4.3.5 Genitalia (Fig. 6H)

Absence of abdominal rods.

Gonocoxite: 223 μ m long, 68 μ m width, with randomly distributed internal setae, without any tuft. Absence of basal gonocoxal lobe.



Figure 6. *Phlebotomus (Lewisius) breyi* n. sp. Holotype male. A: head; B: flagellomeres 1, 2 and 3 (=AIII, AIV and AV); C: labial furca; D: palp; E: third segment of the palp (P3); F: pharynx and cibarium; G: wing; H: genitalia; I: detail of the parameral sheath and the distal part of the genital ducts; and J: gonostyle exhibiting an additional thin basal sixth spine.



Figure 7. *Phlebotomus (Lewisius) breyi* n. sp. Paratype female. A: head; B: mouth parts (from left to right: labrum, hypopharynx, mandible, maxilla, and labial furca); C: palp; D: third segment of the palp; E: flagellomeres 1, 2 and 3 (=AIII, AIV and AV); F: wing; G: furca and bases of spermathecal ducts; and H: spermathecae.

Gonostyle: 117 μ m long with 5 thick spines (two terminal ones, the superior external implanted subapically, the inferior external situated in the apical third and the internal in its middle). Presence on the holotype, as well as on another specimen, of a gonostyle exhibiting an additional thin basal sixth spine (Fig. 6J).

Absence of accessory setae.

Simple paramere 189 μ m long with a slight tubercle carrying about 6 setae on its lower side.

Absence of accessory spine between the paramere and the parameral sheath.

Parameral sheath: 100 μ m straight, with a blunt end at its top (Fig. 61).

Aedeagal ducts: 561 μ m long, isodiametric, pointed at their top. Sperm pump 108 μ m long. Ejaculatory apodeme 96 μ m long.

Epandrial lobes: 225 μ m long, about as long as the gonocoxites, without permanent setae.

3.5.1 Description of the female *Ph. breyi* n. sp. paratype (specimen LAOS#251 type1) (Fig. 7)

3.5.1.1 Head (Fig. 7A)

Occiput with several lines forming a thick stripe in the posterior occiput and a narrow line along the superior part of the eyes.

Clypeus 178 μm long, exhibiting 47 setae. Anterior limit difficult to observe.

Eyes: 223 µm long, 128 µm wide.

Incomplete interocular sutures.

Flagellomeres (Fig. 7E) f1 (=AIII) = 293 μ m, f2 (=AIV) = 129 μ m, f3 (=AV) = 126 μ m.

Flagellomere 1 longer than $f^2 + f^3$.

Presence of 2 short ascoids never reaching the next articulation from f1 to f13.

Ascoidal formula: 2/f1 - f13.

Ascoid/f2 length ratio: 0.57

One distal papilla on f1 and f2 (Fig. 7E). Lack of papilla from f3 to f11. One papilla on f1. One on f2. No papilla on f3 to f10. One papilla on f11. Five on f12 and f13. And four on f14.

No simple seta from f1 to f7. Presence of one or several simple setae from f9 to f14.

Palps (Fig. 7C): p1 = 51 μ m, p2 = 213 μ m, p3 = 218 μ m, p4 = 173 μ m, p5 = 413 μ m.

Palpal formula: 1, 4, (2, 3), 5

Presence of about 15 club-like Newstead's sensilla on p3 (Fig. 7D). No Newstead's sensillum on other palpal segments.

Presence of one simple seta on distal p3; seven on p4; about 40 on p5. No simple seta on p1 and p2.

Labrum 309 μ m long. Limit between the labrum and the clypeus difficult to observe.

Labial furca closed (Fig. 7B).

Cibarium armed with many tiny teeth pointed backwards.

Little pharyngeal teeth, commonly dot-like, sometimes pointed and oriented backwards. All teeth are arranged along parallel curved lines.

Absence of sclerotized area.

3.5.1.2 Cervix

Two cervical sensilla. Ventro-cervical sensilla not observed.

3.5.1.3 Thorax

654 µm long.

Light brown sclerites.

Mesonotum: post-alar setae non-observed.

Pleurae: five proepimeral setae; absence of the upper and lower anepisternal, anepimeral, metaepisternal and metaepimeral setae; presence of fine setae on the anterior region of the katepisternum, and absence of the suture between metaepisternum and katepimeron. Metafurca mounted in lateral view on all specimens.

Wings (Fig. 7F): length = 1690 μ m; width = 564 μ m. r5 = 1139 μ m, α (r2) = 398 μ m, β (r2 + 3) = 174 μ m, δ = 82 μ m, γ (r2 + 3+4) = 332 μ m, e (r3) = 532 μ m, θ (r4) = 789 μ m. Width/ γ = 1.70.

3.5.1.4 Abdomen

Tergites ii–v: presence of randomly distributed setae. Tergite VIII and IX not observed. Cerci 133 μ m long. Setae were not observed on the X sternite.

3.5.1.5 Genitalia (Figs. 7G and 7H)

Spermathecae were measured and drawn in Marc-André solution on specimen 356-1 before its remounting in CMCP9 medium. Smooth and thin wall individual ducts 450 µm long (Fig. 7H). Ducts are isodiametric except for a basal slight enlargement (Fig. 7G). Annealed spermathecae with 12 or 13 bead-like rings. Terminal knob (head) carried by a long and narrow neck.

Genital fork without lateral apodemes.

3.5.2 Description of *Phlebotomus sinxayarami* Vongphayloth & Depaquit n. sp. (Figs. 8 and 9)

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Genus *Phlebotomus* Rondani & Berté, in Rondani 1840. Subgenus *Lewisius* Depaquit & Vongphayloth n. subg.

Type locality: Pha Nok Kok cave (18°30′ N, 101°59′ E), Feung district, Vientiane province, Laos.

Type specimens: Holotype male (voucher NGS 356-17) deposited at the Laboratory of Entomology of the Muséum National d'Histoire Naturelle de Paris (identification number MNHN-ED-ED11201). Two female and two male paratypes deposited at the Laboratory of Entomology of the Muséum National d'Histoire Naturelle de Paris (identification numbers MNHN-ED-ED11202, MNHN-ED-ED11209, MNHN-ED-ED11210, MNHN-ED-ED11211).

One male and one female paratype deposited at the Natural History Museum of London, UK (identification numbers NHMUK014908974 and NHMUK014908975). One female and one male paratype deposited at the Laboratory of Entomology of Institut Pasteur du Laos.

Etymology: Epithet *sinxayarami* refers to the Sinxayaram temple located close to the cave where the type specimens have been caught.



Figure 8. *Phlebotomus (Lewisius) sinxayarami* n. sp. Holotype male. A: palp; B: pharynx and cibarium; C: flagellomeres 1, 2 and 3 (=AIII, AIV and AV); D: labial furca; E: head; F: third segment of the palp (P3); G: wing; and H: genitalia.

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Figure 9. *Phlebotomus (Lewisius) sinxayarami* n. sp. Paratype female. A: head; B: mouth parts (labrum, hypopharynx, mandible, maxilla, and labial furca from left to right); C: third segment of the palp (P3); D: palp; E: pharynx and cibarium; F: flagellomeres 1, 2 and 3 (=AIII, AIV and AV); G: spermathecae; H: furca and bases of spermathecal ducts; and I: wing.

Note: The authors of the new taxa are different from the authors of this paper: Article 50.1 and Recommendation 50A of the International Code of Zoological Nomenclature [24].

3.5.3 Description of the male *Ph. sinxayarami* n. sp. holotype (specimen NGS 356-17)

3.5.3.1 Head (Fig. 8E)

Occiput with several lines forming a thick stripe in the posterior occiput and a narrow line along the superior part of the eyes.

Clypeus 122 μ m long, exhibiting 35 setae. Anterior limit difficult to observe.

Eyes 183 µm long, 95 µm wide.

Complete interocular sutures.

Flagellomeres (Fig. 8C) f1 (=AIII) = 342 μ m, f2 (=AIV) = 148 μ m, f3 (=AV) = 147 μ m.

Flagellomere 1 longer than f2 + f3.

Presence of two very short ascoids never reaching the next articulation from f1 to f9, 1 from f10 to f11, and no ascoid on f12 to f14.

Ascoidal formula: 2/f1-f9, 1/f10-f11, 0/f12-f14.

Length ratio: Ascoid/f2 = 0.23

One distal papilla on f1 and f2 (Fig. 8C). Lack of papilla from f3 to f11. One papilla on f1 and f2. Three on f11. Four papillae on f12, five papillae on f12 to f14.

No simple setae from f1 to f7. Presence of one or several simple setae from f8 to f14.

Palps (Fig. 8A): $p1 = 37 \mu m$, $p2 = 108 \mu m$, $p3 = 122 \mu m$, $p4 = 86 \mu m$, $p5 = 270 \mu m$.

Palpal formula: 1, 4, 2, 3, 5

Presence of 8 club-like Newstead's sensilla on p3 (Fig. 8F). No Newstead's sensillum on other palpal segments.

Presence of one simple seta on distal p3; four simple setae on p4; about 15 on p5. No simple seta on p1 and p2.

Labrum 191 μm long. Limit between the labrum and the clypeus difficult to observe.

Labial furca closed (Fig. 8D).

Cibarium armed with many tiny dot-like teeth, difficult to observe, despite the use of phase contrast (Fig. 8B).

Little pharyngeal teeth, commonly dot-like, sometimes pointed, and oriented backwards. All teeth are arranged along parallel curved lines.

Absence of sclerotized area.

3.5.3.2 Cervix

Two cervical sensilla.

Ventro-cervical sensilla not observed.

3.5.3.3 Thorax

513 µm long.

Light brown sclerites.

Mesonotum: post-alar setae non-observed.

Pleurae: five proepimeral setae; absence of the upper and lower anepisternal, anepimeral, metaepisternal and metaepimeral setae; presence of fine setae on the anterior region of the katepisternum, and absence of the suture between metaepisternum and katepimeron. Metafurca mounted in lateral view on all specimens. Wings (Fig. 8G): length = 1619 μ m; width = 475 μ m. $r5 = 1156 \ \mu$ m, α (r2) = 434 μ m, β (r2 + 3) = 149 μ m, δ = 108 μ m, γ (r2 + 3 + 4) = 298 μ m, e (r3) = 572 μ m, θ (r4) = 814 μ m. Width/ γ = 1.60.

3.5.3.4 Abdomen

Tergites ii–v: presence of randomly distributed setae. Tergites ii–vii: absence of tergal papillae.

3.5.3.5 Genitalia (Fig. 8H)

Absence of abdominal rods.

Gonocoxite: 204 μ m long, 58 μ m width, with randomly distributed internal setae, without any tuft. Absence of basal gonocoxal lobe.

Gonostyle: 105 μ m long with 5 thick spines (two terminal ones, two median ones and an intermediate one).

Absence of accessory setae.

Simple paramere: 140 μ m long with a slight tubercle carrying about 5 setae on its lower side.

Absence of accessory spine between the paramere and the parameral sheath.

Parameral sheath: 100 µm straight, rounded at its top.

Aedeagal ducts, 390 μm long, isodiametric, pointed at their top. Sperm pump 98 μm long. Ejaculatory apodeme 96 μm long.

Epandrial lobes: 196 μm long, about as long as the gono-coxites, without permanent setae.

3.5.4 Description of the female *Ph. sinxayarami* n. sp. paratype (specimen NGS 356-29) (Fig. 9)

3.5.4.1 Head (Fig. 9A)

Occiput with several lines forming a thick stripe in the posterior occiput and a narrow line along the superior part of the eyes.

Clypeus 138 μ m long, exhibiting 35 setae. Anterior limit difficult to observe.

Eyes 179 μm long, 97 μm wide.

Complete interocular sutures.

Flagellomeres (Fig. 9F) f1 (=AIII) = 301 μ m, f2 (=AIV) = 121 μ m, f3 (=AV) = 112 μ m.

Flagellomere 1 longer than $f^2 + f^3$.

Presence of 2 short ascoids never reaching the next articulation from f1 to f11, probably two but the second one is not visible from f12 and no ascoid on f13–f14.

Ascoidal formula: 2/f1–f11, 1(1)/f12, 0/f13.

Ascoid/f2 length ratio: 0.56

One distal papilla on f1 and f2. No papilla from f3 to f9. One on f10. Three on f11 to f13. Four papillae on f12, four on f13.

No simple seta from f1 to f7. Presence of one or several simple setae from f9 to f14.

Palps (Fig. 9D): $p1 = 37 \mu m$, $p2 = 120 \mu m$, $p3 = 128 \mu m$, $p4 = 923 \mu m$, $p5 = 238 \mu m$.

Palpal formula: 1, 4, (2, 3), 5

Presence of about 15 club-like Newstead's sensilla on p3 (Fig. 9C). No Newstead's sensilla on other palpal segments.

Presence of one simple seta on distal p3; seven simple setae on p4; about 40 on p5. No simple seta on p1 and p2.

Labrum 214 μ m long. Limit between the labrum and the clypeus difficult to observe.

Labial furca closed (Fig. 9B).

Cibarium armed with many tiny teeth pointed backwards (Fig. 9E).

Little pharyngeal teeth, commonly dot-like, sometimes pointed and oriented backwards. All teeth are arranged along parallel curved lines.

Absence of sclerotized area.

3.5.4.2 Cervix

Two cervical sensilla. Ventro-cervical sensilla not observed.

3.5.4.3 Thorax

521 µm long.

Light brown sclerites.

Mesonotum: post-alar setae non-observed.

Five proepimeral setae.

Pleurae: five proepimeral setae; absence of the upper and lower anepisternal, anepimeral, metaepisternal and metaepimeral setae; presence of fine setae on the anterior region of the katepisternum, and absence of the suture between metaepisternum and katepimeron. Metafurca mounted in lateral view on all specimens.

Wings (Fig. 91): length = 1752 μ m; width = 588 μ m. $r5 = 1266 \ \mu$ m, α (r2) = 535 μ m, β (r2 + 3) = 167 μ m, δ = 163 μ m, γ (r2 + 3+4) = 304 μ m, e (r3) = 674 μ m, θ (r4) = 926 μ m, π = 0 μ m. Width/ γ = 1.93.

3.5.4.4 Abdomen

Tergites ii–v: presence of randomly distributed setae. About 20 setae on tergite VIII. Absence of protuberance on tergite IX. Cerci 118 µm long. Setae not observed on the X sternite.

3.5.4.5 Genitalia (Figs. 9G and 9H)

Spermathecae were measured and drawn in Marc-André solution on specimen 356-1 before its remounting. Smooth and thin wall individual ducts 160 µm long. Ducts are isodiametric and enlarged at their bases (Fig. 9H). Annealed spermathecae with 14 or 15 bead-like rings. Terminal knob (=head) inserted in a wide neck (Fig. 9G).

Genital fork with short lateral apodemes.

Tables 4 and 5 summarize the measurements carried out on several male and female specimens of *Ph. breyi* n. sp., *Ph. sinx-ayarami* n. sp., and *Ph. betisi*.

4 Discussion

The new species status given to *Ph. breyi* n. sp. and *Ph. sinxayarami* n. sp. is very strong. It is based on morphologic, morphometric, geomorphometric, molecular and proteomic arguments even though this last approach was not possible on the specimens of *Ph. betisi* in our possession that were all preserved in alcohol before being mounted. The association of males and females, in addition to the fact that each of the specimens are sympatric, having all been captured in the same cave, is based on the same evidence, regardless of the approach considered.

From a morphological point of view, the three species are easily individualized.

In both sexes, *Ph. betisi* and *Ph. breyi* do not have a complete interocular suture while *Ph. sinxayarami* owns one. This character is very unusual in the genus *Phlebotomus* [38] and is rather observed in the American genera *Warileya* [18]. In the Old World, the genus *Chinius* also shares a complete interocular suture [15].

For males, the main discriminating character (Tables 4, 5 and 6) between these three species is the f1/f2 + 3 ratio. This ratio is low in *Ph. breyi* n. sp., intermediate in *Ph. sinxayarami* and high for *Ph. betisi*. There is no overlap between the three species for this trait. Moreover, the length of the aedeagal ducts is discriminating: they are very long in *Ph. breyi* (>478 µm), long in *Ph. sinxayarami* (331–470 µm) and short in *Ph. betisi* (311–387 µm). Similarly, the palps (particularly p4 and p5) are short in *Ph. sinxayarami* while they are long in *Ph. breyi* and *Ph. betisi*.

For females, the length of the palps, particularly p4 and p5 are short in *Ph. Sinxayarami*, while they are long in *Ph. breyi* and *Ph. betisi*. The spermathecal ducts of *Ph. sinxayarami* are longer than those of *Ph. breyi*. The distal part of the spermatheca bearing the head is similar to a neck and thin in *Ph. breyi* and in *Ph. betisi*, while it is enlarged in *Ph. sinxayarami*.

Until now, Ph. betisi was the only Phlebotomus from Southeast Asia to exhibit in the male a gonostyle bearing five spines associated with a simple paramere, differing from the Phlebotomus of the subgenus Euphlebotomus having a fivespine gonostyle associated with a complex paramere. Females were the only ones of the genus Phlebotomus in this same region to have a pharyngeal framework consisting of small punctiform teeth and annealed spermathecae whose head is carried by a neck like that observed in females of the subgenus Larroussius. This exclusivity in the species described so far prompted the authors to quickly identify the specimens possessing these characters as being Ph. betisi. Paradoxically, this great originality of the characters may have hidden specific diversity which would henceforth prompt us to review all the mentions of Ph. betisi in the literature, starting with those having been used to describe the males, although separated only by about 20 kilometres from the type-locality in Malaysia. A checking of the specimens recorded in Thailand [44] or Vietnam [48, 49] is now necessary. Additionally, several records of Ph. major in Southeast Asia [5-7, 23, 32-35, 44] are probably irrelevant and certainly refer to Ph. betisi. We recommend their re-examination.

Interestingly, both males and females did not exhibit any papilla on the 3rd flagellomere. This is completely unusual regarding the genus *Phlebotomus*. If old descriptions did not mention the record/absence of such papilla on the 3rd flagellomere, recent observations carried out on the genera *Phlebotomus* and *Sergentomyia* highlighted every time the presence of such papilla on the 3rd flagellomere for members of the genus *Phlebotomus*, whereas the species belonging to the genus *Sergentomyia* never exhibits such papilla [11, 13, 16, 39, 40]. This unusual observation means that all species belonging to the subgenus *Lewisius* n. subg. share a character previously observed in the genus *Sergentomyia*.

The position of *Ph. betisi*, *Ph. breyi*, and *Ph. sinxayarami* in the subgenus *Larroussius* does not seem relevant to us. When describing the first species, Lewis & Wharton [29] did not

		Ph	. <i>breyi</i> n.	sp.			Ph. sin	xayarami	n. sp.		Ph. betisi					
-	No. of specimens	Max	Min	Average	Standard deviation	No. of specimens	Max	Min	Average	Standard deviation	No. of specimens	Max	Min	Average	Standard deviation	
Wing																
Length	9	2164.33	1695.71	2000.31	160.08	10	2239.45	1707.52	1970.33	147.90	2	2098.49	1928.28	2013.39	120.36	
Width	9	696.96	565.15	640.79	46.95	10	658.73	490.26	598.20	48.96	3	670.45	624.79	640.07	26.31	
Alpha (R2) α	9	557.30	387.67	469.89	57.09	10	591.52	397.13	514.80	54.60	3	600.14	510.61	552.44	45.05	
R3	9	730.64	533.28	640.46	65.00	10	930.24	547.78	682.38	99.96	3	757.67	658.38	716.25	51.65	
R4	9	1048.92	799.16	931.16	80.57	10	1050.49	804.78	947.41	67.15	3	1007.40	897.49	960.02	56.50	
R5	9	1489.49	1124.37	1342.68	117.66	10	1482.92	1141.07	1304.20	91.42	3	1417.85	1306.74	1378.02	61.87	
Beta (R2 + R3) β	9	226.07	169.76	201.51	17.55	10	243.85	158.83	203.36	30.46	3	183.02	153.49	171.82	16.01	
Delta (R2 + 3-R1) δ	9	167.29	72.67	112.23	33.21	10	182.15	52.49	110.00	34.96	3	215.31	168.39	194.75	23.99	
Gamma (R2 + 3+4)	9	440.33	321.69	384.19	40.27	10	408.74	275.87	322.91	38.15	3	409.27	374.13	388.13	18.63	
Pi (R2 + 3-M1 + 2)	9	59.22	9.51	29.13	15.87	10	113.23	20.18	71.62	30.97	3	66.06	32.29	52.09	17.62	
R2/R2 + 3	9	2.63	1.93	2.34	0.23	10	3.26	2.02	2.58	0.44	3	3.35	2.99	3.22	0.20	
Head																
f1 (AIII)	5	293.87	256.01	276.80	14.95	10	346.48	253.05	310.18	25.91	3	352.87	309.06	328.91	22.19	
f2 (AIV)	5	134.90	113.78	123.19	8.33	10	136.21	105.74	122.48	8.25	3	143.06	132.07	136.65	5.72	
f3 (AV)	4	125.56	111.63	120.07	6.35	10	137.78	109.65	124.17	8.08	3	135.18	128.70	132.30	3.30	
f2 + f3 (AIV + AV)	4	260.46	225.41	244.22	15.48	10	273.99	215.39	246.65	16.17	3	278.24	260.77	268.96	8.79	
f1/f2 + f3 (AIII/	4	1.20	1.11	1.14	0.04	10	1.33	1.17	1.26	0.05	3	1.27	1.19	1.22	0.04	
AIV + AV)																
Clypeus	9	182.93	130.08	167.78	17.08	10	143.69	123.45	132.39	5.70	3	161.20	153.89	157.39	3.66	
Labrum	9	320.26	275.54	304.78	16.33	10	284.85	205.14	249.16	23.52	3	311.61	293.77	300.25	9.87	
F1 (AIII)/labrum	5	0.92	0.87	0.89	0.02	10	1.38	1.12	1.25	0.08	3	1.19	1.04	1.10	0.09	
P1	10	60.21	39.45	50.31	6.75	10	39.07	33.04	36.37	2.16	3	55.68	45.35	49.27	5.59	
P2	10	218.32	165.30	194.29	17.17	10	155.27	112.01	135.61	11.18	3	215.06	193.45	205.74	11.11	
P3	10	216.12	173.41	195.10	13.32	10	147.11	121.12	135.82	9.88	3	209.92	178.74	198.40	17.11	
P4	10	172.48	141.01	154.12	12.74	10	105.16	80.58	92.03	8.11	3	140.66	126.39	135.84	8.18	
P5	9	426.75	308.15	372.52	47.93	10	293.55	135.30	212.32	44.45	3	452.85	393.31	428.49	31.21	

Table 4. Measurement of females of three species: Ph. breyi n. sp., Ph. sinxayarami n. sp., and Ph. betisi from Malaysia.

		Pł	h. bre yi n.	sp.			Ph.	sinxayara	<i>mi</i> n. sp.			Ph. betisi				
-	No. of specimens	Max	Min	Average	Standard deviation	No. of specimens	Max	Min	Average	Standard deviation	No. of specimens	Maxi	Min	Average	Standard deviation	
Wing																
Length	9	1968.57	1740.77	1877.49	77.67	7	1721.45	1547.89	1649.50	60.22	8	1744	1596	1683	55.7	
Width	10	631.18	546.19	588.13	26.28	7	544.03	461.90	500.79	25.11	9	522	483	504	13.3	
Alpha (R2) a	10	472.32	391.64	439.02	24.10	7	445.45	388.89	419.55	21.82	9	472	401	435	21.3	
R3	10	645.88	560.07	591.24	28.71	7	589.38	512.09	557.18	25.50	_	_	-	-	_	
R4	10	916.34	827.67	855.10	29.01	7	864.86	757.65	805.96	37.81	_	_	-	-	_	
R5	10	1321.36	1225.78	1259.27	36.48	7	1172.98	1045.51	1116.89	45.16	_	_	_	_	_	
Beta $(R2 + R3)$ b	10	200.64	170.93	183.35	8.95	7	178.96	146.72	165.82	13.39	9	155	113	135	14.4	
Delta d $(R2 + 3 - R1)$	10	151.00	64.64	102.59	24.25	7	91.54	45.77	73.27	17.75	9	298	96	164	63.6	
Gamma $(R2 + 3 + 4)$ g	10	410.62	331.05	370.65	21.36	7	304.14	227.50	274.38	25.60	9	455	279	334	52.6	
Pi p $(R2 + 3-M1 + 2)$	10	63.34	6.73	29.63	14.81	7	86.21	33.64	57.36	20.17	9	103	14	54	30.9	
R2/R2 + 3	10	2.57	2.09	2.40	0.14	7	2.90	2.35	2.54	0.19	_	_	_	_	_	
Head																
f1 (AIII)	8	330.98	268.11	303.08	21.33	7	334.08	239.33	301.88	34.88	10	347	298	328	15.6	
f2 (AIV)	7	146.28	130.56	137.93	6.65	7	149.48	122.03	135.36	9.41	10	153	142	148	4.5	
f3 (AV)	7	141.93	128.48	135.08	5.15	7	144.94	119.95	134.97	8.05	10	149	135	142	4.8	
f2 + f3 (AIV + AV)	7	286.02	259.70	273.01	10.54	7	294.42	241.98	270.33	17.27	10	302	278	290	9.01	
Clypeus	10	164.73	142.70	154.60	6.71	7	125.67	109.15	119.94	5.26	_	_	_	_	_	
Labrum	10	261.69	238.06	250.50	8.65	7	213.48	192.18	202.89	7.60	10	267	208	242	18.9	
f1/f2 + f3 (AIII/	7	1.18	1.03	1.11	0.05	7	1.23	0.88	1.12	0.12	10	1.17	1.07	1.13	0.03	
AIV + AV																
f1 (AIII)/labrum	10	1.31	0.00	0.97	0.52	7	1.69	1.21	1.49	0.17	10	1.52	1.18	1.36	0.1	
P1	9	53.09	36.97	43.47	4.86	8	35.31	30.84	32.52	1.51	10	60	34	45	7.6	
P2	9	186.87	136.69	174.76	15.10	8	129.24	101.22	115.96	9.53	10	215	150	195	21.7	
P3	8	188.41	174.74	180.33	4.86	8	130.99	103.68	119.59	9.41	9	232	165	182.3	19.9	
P4	8	153.53	136.26	142.99	6.41	8	93.46	66.40	84.05	8.07	9	121	99	108.1	7.5	
P5	6	424.41	292.33	364.61	54.43	8	272.04	182.64	233.49	32.05	9	380	308	348	27.4	
Genitalia																
Parameral sheath	10	106.28	96.92	101.13	2.32	8	85.59	74.00	79.99	4.19	9	133	93	111	11.7	
Gonostyle	10	117.08	103.20	111.74	4.00	8	111.54	99.20	103.98	4.04	9	117	102	109	4.3	
Gonocoxite	10	229.16	207.78	220.34	7.39	8	204.76	183.86	196.20	8.06	9	224	168	193	15.7	
Sperm pump (SP)	10	116.51	95.83	107.49	6.32	8	110.63	92.17	100.76	5.55	9	129	112	121	4.8	
Paramere	10	196.99	160.00	182.62	12.92	8	153.32	138.06	142.47	4.92	9	187	153	170	10.3	
Surstyle	10	237.98	198.33	216.01	13.85	8	195.37	172.85	187.13	7.84	9	218	179	198	12	
Aedeagal ducts (AD)	10	655.39	535.60	599.87	34.11	8	411.75	328.91	374.47	26.02	9	470	331	362	39.7	
Gonocoxite/gonostyle	10	2.15	1.88	1.97	0.10	8	2.00	1.81	1.89	0.07	10	2.02	1.44	1.78	0.17	
AD/SP	10	6.25	5.03	5.59	0.39	8	4.02	3.35	3.72	0.26	10	3.92	2.74	3.01	0.35	

Table 5. Measurement of males of three species: Ph. breyi n. sp., Ph. sinxayarami n. sp., and Ph. betisi from Malaysia

he three presently known species	of Lewisius n. subg.	
Ph. betisi Lewis & Wharton	Ph. sinxayarami n. sp.	Ph. breyi n. sp.
2/f1-f13, 0/f14	2/f1-f13, 0/f14	2/f1-f13, 0/f14
Presence on f1–f2, f9–f14, and absence on f3–f8	Presence on f1-f2, f10-f14, and absence on f3-f9	Presence on f1–f2, f12–f14, and absence on f3–f11
> 1	> 1	< 1
< 141 (126–141)	< 141 (80–110)	> 141 (141–173)
about 20 clearly separated rings	about 12-13 clearly separated	about 14-15 clearly separated

rings with short neck < 400, No

2/f1-f9, 1/f10-f11, 0/f12-f14

Presence on f1-f2, f10-f14, and

common duct

absence on f3-f9

< 500 um

< 4

Table 6. Differential diagnosis of the three pr

with long neck < 400, No

2/f1-f6, 1/f7-f12, 0/f13-f14

Presence on f1-f2, f9-f14, and

common duct

absence on f3-f8

< 500

< 4

Gender and character

antennal papillae

f1 (AIII)/labrum

palp segment 4

length of spermathecal individual

spermathecae

ascoid formula

antennal papillae

Aedeagal ducts

aedeagal ducts/sperm pump

ducts

Males

Females ascoid formula

classify Ph. betisi in the subgenus Larroussius explaining about
its systematic position: "the spermatheca shows that this species
may belong to the group of Ph. major Annandale (subgenus
Larroussius of Theodor, 1948, 1958), but the bead-like seg-
ments and narrowness of the process are unusual. The discov-
ery of the male would probably clarify the position of Ph.
betisi". In 1978, Lewis [28] initially classified Ph. betisi in
the subgenus Larroussius then followed by several authors
reviewing the classification of sandflies [8, 9, 27, 43]. When
they described the male, Khadri et al. [25] indicated that within
the Larroussius subgenus, two distal and three median spines
on the style were usually present. Phlebotomus betisi differed
from other Larroussius males by the position of these spines:
two distal, one between these and the two intermediate ones.
This character is sometimes shared by <i>Phlebotomus</i> belonging
to the subgenus <i>Euphlebotomus</i> . These morphological charac-
ters could thus be sufficient to support the validity of the sub-
genus Lewisius n. subg. Moreover, the molecular approach
(Fig. 2) very clearly individualizes the three species of <i>Lewisius</i>
revealing the monophyly of this new subgenus. Molecular
phylogeny based on ML analysis of mtDNA cyt b sequences
also separates <i>Larroussius</i> from <i>Lewisius</i> very clearly. One
ing a robust molecular phylogeny including such veried
a robust molecular phylogeny including such valied
phlabotomus Artemianus and Swaphlabotomus are doubtful
and the apparent paraphyly of <i>Paraphlebotomus</i> in the present
study contrasts with the robust monophyly obtained on gen-
ome-wide data it was possible to individualize the subgenus
Artemievus by excluding Ph alexandri of Paraphlebotomus
[12]. Our goal was not to reconstruct a phylogeny of the genus
<i>Phlebotomus</i> but to test the monophyly of <i>Lewisius</i> as well as
its relationships with the <i>Larroussius</i> . Moreover, the <i>Lewisius</i>
has the subgenus <i>Madaphlebotomus</i> as sister group. This rela-
tionship between Phlebotomine sandflies from Madagascar and
from Southeast Asia deserves to be explored in the light of
more conserved molecular markers.

Proteomic data, based on MALDI-TOF mass spectrometry, are consistent with the molecular analysis. With this technique, a high distance separates spectra from specimens of Larroussius subgenus and those of the subgenus Lewisius. Use of MALDI-TOF is also very promising for identification of the two newly described species Ph. breyi and Ph. sinxayarami. No fresh specimens of Ph. betisi were, however, available for MALDI-TOF analysis, and thus did not allow us to fully test the discrimination capabilities of this technique. The constitution of a MALDI-TOF spectral database for Asian sandflies would be particularly interesting for rapid and inexpensive screening.

Morphometric data are coherent with molecular and proteomic approaches. Differences in sizes of wings between male populations are significant. Nonetheless, the morphometric evidence of this current work does not provide an unequivocal result. Principal Component Analysis of the females showed low segregation, whereas males of the two species were more easily separated.

As a side note concerning the biology of this species, it is worth mentioning that our specimen was captured at the entrance of the cave and that we detected a blood meal from a water buffalo in its abdomen. These buffalo were present in a rice field located below the cave at a distance of about 100 to 200 meters from the entrance. They could not access the cave. This trophic preference thus indicates that Ph. sinxayarami females have to leave the cave in order to take their blood meals outside, and therefore do not remain permanently inside the cave.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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rings with long neck > 400, No

2/f1-f9, 1/f10-f11, 0/f12-f14

Presence on f1-f2, f12-f14, and

common duct

absence on f3-f11

> 500 µm> 4

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Supplementary material

The Supplementary materials of this article are available at https://www.parasite-journal.org/10.1051/parasite/2023021/olm.

Figure S1: Boxplot of centroid sizes of wings landmarks of *Phlebotomus (Lewisius) breyi* n. sp. and *Phlebotomus (Lewisius) sinxayarami* n. sp. for male and female specimens.

Figure S2: Deformation grids of wings landmarks of *Phlebotomus* (*Lewisius*) *breyi* n. sp. and *Phlebotomus* (*Lewisius*) *breyi* n. sp. for males and females using *Ph. betisi* as reference.

Figure S3: Representative MALDI-TOF spectra of Ph. sinxayarami n. sp. (A, C) and Ph. breyi n. sp. (B).

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