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A novel geographic population of *Dasyhelea ludingensis* Zhang & Yu from Guizhou Province, China (Diptera: Ceratopogonidae)

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Abstract

The study presents a report on the intraspecific variation among female adults of *Dasyhelea ludingensis* Zhang & Yu. Specimens were collected using a Malaise trap near a pond in Honghuagang District, Zunyi City, Guizhou Province, China. Morphological structures were examined utilizing both optical microscopy and scanning electron microscopy. The partial mitochondrial Cytochrome oxidase I (COI) was used to analyze the genetic relationships among samples. *Dasyhelea ludingensis* should be classified as a species with significant intraspecific morphological variation, as supported by the molecular and morphological data.

Key words: Intraspecific variation, COI gene, Ultrastructure, Integrative taxonomy, Phylogenetic analysis

Introduction

Phenotype can be influenced by genetic factors and/or environmental conditions (Song *et al.* 2009), leading to intraspecific morphological variation. The variation is frequently observed in many insect species, such as dragonfly *Erythrodiplax Media* (Pires *et al.* 2021), blood-biter *Forcipomyia* (*Lasiohelea*) *taiwana* (Shih *et al.* 2019), or species groups, such as ants (Noh *et al.* 2020; Tomkins & Moczek 2009), bees (Duncan *et al.* 2022; Kim *et al.* 2019), termites (Song *et al.* 2009), aphids (Watanabe *et al.* 2016) and so on. Morphological variation poses a challenge in traditional insect taxonomy, as species identification depends mainly on morphological characteristics and is often based on a small number of samples (Li *et al.* 2019). Consequently, it is inevitable that the morphological variation of the species cannot be comprehensively described or leading to synonyms (Zhao *et al.* 2023).

The limitations of traditional taxonomy lead to emergence of genomic approaches to taxon diagnosis that exploit diversity among DNA sequences to identify organisms (Nagpure *et al.* 2012), such as DNA barcoding. The use of DNA barcoding for species identification solves many of the problems associated with the application of morphology-based approaches (Layton *et al.* 2016).

Midges of the genus *Dasyhelea* Kieffer, 1911, a member of the family Ceratopogonidae with 628 extant species in the global fauna, exhibit a wide distribution and can be found in various moist habitats (Borkent & Dominiak 2020; Borkent *et al.* 2022). Despite the extensive nature of this genus, it has been neglected for its non-bloodsucking behavior (Dominiak & Salmela 2023). Nonetheless, some researchers have noted that certain species within this genus play a role in the pollination of cocoa trees (Theobroma cacao L.) and rubber trees (Hevea brasiliensis) (Lee *et al.* 1989), and there is an increased emphasis on the beneficial ecological interactions of these midges (Yu *et al.* 2005).

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Dasyhelea ludingensis was first described by Zhang and Yu based on one female adult in 1996. It was revised by Yu *et al*. (2005). In this paper, we describe a new geographic population of *D. ludingensis* from Guizhou Province using morphological data from optical microscopy and scanning electron microscopy. A phylogenetic tree based on partial COI genes was constructed to discuss its systematic position.

Material and methods

2.1. Specimen Collection

The specimens were collected with Malaise traps near a pond in Zunyi City, Guizhou Province (18°15'–27°19'N, 100°15'–120°12'E) during the period of May 2022 to October 2023. Subsequently, all collected specimens were preserved in 95% ethanol in a 4°C freezer for further study.

2.2. Specimen Preparation for Microscope

Preliminary identifications were assigned under an Olympus SZX7 stereomicroscope and the dorsal view of the scutum was immediately photographed with an Olympus DP22 camera attached to the same microscope. Subsequently, specimens were mounted under separate cover slips on microscope slides by the methods described in Yu *et al.* (2005). Photomicrographs were taken with an Olympus BX43 microscope equipped with an Olympus DP27 camera and stacked images were obtained using the Helicon Focus 8.1.0 (Helicon Soft Ltd). All the photomicrographs were edited using Adobe Photoshop 2023. The morphological terms as described by Zhang and Yu (1996) were followed. In this paper, 22 specimens were measured for each character (n = number of specimens examined).

2.3. Specimen Preparation for SEM

The following preprocessing steps were performed: specimens were washed in 0.1M PBS for 10 minutes twice, followed by fixation in 2% Glutaraldehyde for 2 hours, washing four times with PBS for 10 minutes each, and fixation with 1% osmic acid solution for 40 minutes. In accordance with the methodology described by Jiang *et al.* (2018), the specimens underwent dehydration using gradient of ethanol concentrations (30%, 50%, 70%, 90%, and 100%) for 15 minutes each. Subsequently, the specimens were subjected to critical point drying, and finally were coated with gold before being examined using a Scanning Electron Microscope SU8010 (Hitachi High-Technologies Corporation, Tokyo, Japan).

2.4. DNA Extraction, PCR Amplification and Sequencing

Total genomic DNA was extracted for each dissected midge from the body using a TIANamp Genomic DNA Kit (Tiangen Biotech (Beijing) Co., Ltd., China), following the manufacturer's instructions, with a final elution volume of 40 µl. The extracted DNA was stored at −20°C until further processing.

The initial DNA fragments for the COI gene were amplified by PCR from the total genomic DNA, respectively. The universal primers used for amplification were LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.* 1994). PCR was performed in a final volume of 25 µl containing 12.5 µl of 2×premix Taq (Takara, Takara Biomedical Technology (Beijing) Co., Ltd., China), 0.4 µM of each primer, and 2 µl of total genomic DNA. After an initial 3 min denaturing step at 94 °C, 36 cycles of amplification were performed with 30 sec at 94 °C, 30 sec at 50 °C, and 1.0 min at 72 °C, and then followed by a final extension for 10 min at 72 °C. The target bands of PCR (about 710 bp) were gel-purified by using TIANquick Midi Purification Kit (Tiangen Biotech (Beijing) Co., Ltd., China), and them were cloned into the pMD19-T Vector (Takara, Takara Biomedical Technology (Beijing) Co., Ltd., China). Three clones were sequenced in both directions with forward and reverse universal primers by Sangon Biotech Co., Ltd. (Shanghai, China). Sequences were deposited to GenBank with the accession numbers from PP783820 to PP783834 as shown in Table 1.

TABLE 1. Samples used in this study, with GenBank accession numbers.

Types	Figure	Voucher number	COI NCBI accession number
Dasyhelea ludingensis W	Figure 2 A	2023092005	PP783820
		2023092006	PP783821
D. Ludingensis type I	Figure 2 B	2023061702	PP783822
		2023051703	PP783823
		2023081704	PP783824
D. Ludingensis type II	Figure 2 C	2022081702	PP783825
		2022051703	PP783826
		2022071704	PP783827
D. Ludingensis type III	Figure 2 D	2023091301	PP783828
		2023091302	PP783829
D. Ludingensis type IV	Figure 2 E	2023091304	PP783830
		2022071701	PP783831
		2022061703	PP783832
D. Ludingensis type V	Figure 2 F	2023071303	PP783833
		2022061901	PP783834

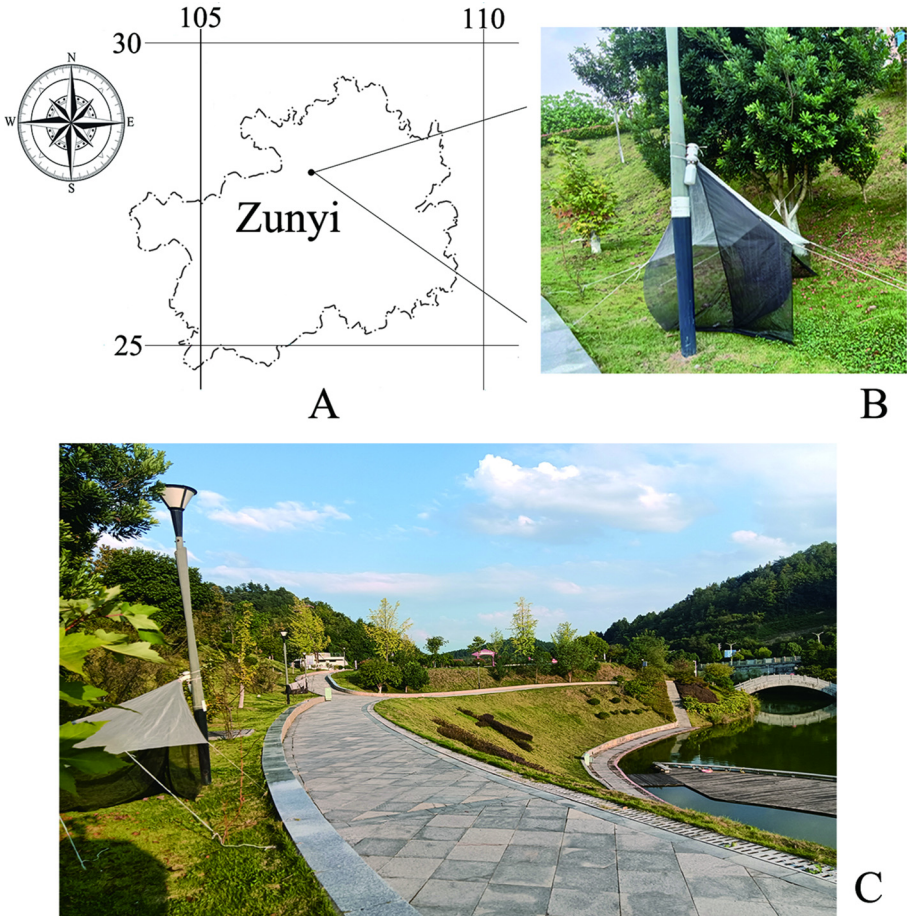

FIGURE 1. Collecting site.

2.5. Genetic distance and phylogenetic Analyses

The genetic distance between each sequence of 18 COI and sequences of *D. ludingensis* were calculated utilizing MEGA 7.0 alignment (Kumar *et al.* 2016). The alignment of nucleotide sequences of 18 COI of the *D. ludingensis* were performed using the ClutalX1.81 software (Plate-Forme de Bio-Informatique, Illkirch, France).

Combining the newly sequenced COI and sequences from NCBI, a total of 19 species from the genus *Dasyhelea* were included in phylogenetic analysis. *Austroconops mcmillani* Wirth & Lee was selected as outgroup (for the full list, see Table 2).

TABLE 2. List of the COI gene analyzed in the present study. List of taxa included in this study.

Order	Family	Genus	Subgenus	Species	Accession No.	Length (bp)
Diptera	Ceratopogonidae	*Austroconops* Wirth & Lee		*Austroconops mcmillani* Wirth & Lee, 1958	KT278281	658
Diptera	Ceratopogonidae	*Dasyhelea* Kieffer	*Dasyhelea*	*Dasyhelea caesia* Remm, 1993	ON342520	658
			Dasyhelea	*Dasyhelea bensoni* Edwards, 1933	HM860912	658
			Dasyhelea	*Dasyhelea baltica* Remm, 1966	ON342003	658
			Dasyhelea	*Dasyhelea malleola* Remm, 1962	ON342124	658
			Dasyhelea	*Dasyhelea flavifrons* (Guérin-Méneville, 1833)	ON341754	658
			Dasyhelea	*Dasyhelea bilineata* Goetghebuer, 1920	ON342131	658
			Prokempia	*Dasyhele dampfi* Kieffer, 1925	ON342505	658
			Prokempia	*Dasyhelea biunguis* Kieffer, 1925	MZ627220	658
			Sebessia	*Dasyhelea holosericea* (Meigen, 1804)	ON341885	658
			Sebessia	*Dasyhelea acuminata* Kieffer, 1919	ON341818	658
			Dicryptoscena	*Dasyhelea notata* Goetghebuer, 1920	KT278270	658
			Dicryptoscena	*Dasyhelea modesta* (Winnertz, 1852)	ON342560	658
			Dicryptoscena	*Dasyhelea lucida* Remm, 1968	ON341978	658
			Pseudoculicoides	*Dasyhelea calycata* Remm, 1972	MZ625619	658
			Pseudoculicoides	*Dasyhelea europaea* Remm, 1962	MZ624632	658
			Pseudoculicoides	*Dasyhelea arenivaga* Macfie, 1943	ON342360	658
			Pseudoculicoides	*Dasyhelea turficola* Kieffer, 1925	JN301663	658

.....continued on the next page

TABLE 2. (Continued)

Order	Family	Genus	Subgenus	Species	Accession No.	Length (bp)
			Pseudoculicoides	*Dasyhelea parallela* Remm, 1962	ON341878	658
				Dasyhelea ludingensis Zhang & Yu, 1996	HQ945375	658
				Dasyhelea ludingensis Zhang & Yu, 1996	JF871777	658
				Dasyhelea ludingensis Zhang & Yu, 1996	LC015046	658
				Dasyhelea ludingensis Zhang & Yu, 1996	PP783820~PP783834	658

PhyloSuite v1.2.3 (Xiang *et al.* 2023; Zhang *et al.* 2020) was used to parse and extract COI annotations recorded and create GenBank submission files and organization tables for the COI. The extraction results were imported into MAFFT (Katoh & Standley 2013) for multiple sequence alignment via the L-INS-I strategy. ModelFinder v2.2.0 (Kalyaanamoorthy *et al.* 2017) was used to select the best-fit model via Bayesian information criterion (BIC) for maximum likelihood (ML) and Bayesian inference (BI) analyses. The ML phylogenies were inferred using IQ-TREE v2.2.0 (Nguyen *et al.* 2015) under a GTR+F+I+G4 model for 5000 ultrafast bootstraps (Minh *et al.* 2013). The BI phylogenies were inferred using Mrbayes 3.2.7a under a GTR+F+I+G4 model with two simultaneous runs of 2 million generations, and trees were sampled every 1,000 generations, with the first 25% discarded as burn-in (Ronquist *et al.* 2012). The phylogenetic analyses using Mrbayes 3.2.7a (Ronquist *et al.* 2012) was carried out on the CIPRES Science Gateway (Miller *et al.* 2010) (https://www.phylo.org/, accessed on 24 December 2024). The finalized trees were visualized with the Interactive Tree of Life (Letunic & Bork 2016) (iTOL: http://itol.embl.de, accessed on 25 December 2024) and edited with CorelDraw 2019 (Corel, Corel Corporation, Canada).

2.6. Specimen Deposition

The voucher specimens were deposited in the Insect Collection of Zunyi Medical University, Zunyi City, Guizhou Province, China (ICZU).

Results

Dasyhelea ludingensis Zhang & Yu

Dasyhelea ludingensis Zhang & Yu, 1996, 18(3): 202. Type locality: China: Sichuan, Luding.
Dasyhelea (*Prokempia*) *ludingensis*: Yu *et al*., 2005. Ceratopogonidae of China (Insecta, Diptera): 213.

3.1. Redescription

Diagnosis. *Female*: Body pale brown to dark brown. Head dark brown. Scutum dark brown uniformly (Figure 2 A) or with yellow lines (Figure 2 B), spots (Figure 2 C) or areas (Figure 2 D–F). Scutellum yellow-green, postscutellum pale brown. Halteres light brown, with white knob. Abdomen brown, membranes between sclerites and abdominal segments yellow. Legs light brown.

Description. ***Female***: Eyes pubescent (Figure 3 A, 4 A–C), separated by less than 1 facet (Figure 3 B, 4 A, C). Frontal sclerite rhombic, with long processes (Figure 3 B, Figure 4 A). Antenna with 13 flagellomeres, without sculptured; flagellomeres cylindrical, with sensilla chaetica, sensilla trichodea and sensilla basiconica, terminal

flagellomere without apical papilla (Figure 3 A, 4 D–F); AR 0.75–0.87 (0.80, n = 22). Apex of labrum with sensilla trichodea (Figure 4 G). Maxillary palp with 5 segments (Figure 3 C); segment 3 cylindrical, with 6–8 capitates sensillae scattered on the basal half surface (Figure 4 H); segment 5 subcylindrical, with long setae (Figure 4 I); PR 2.13–3.33 (2.87, n = 22). Clypeus with 3 pairs of setae (Figure 3 C).

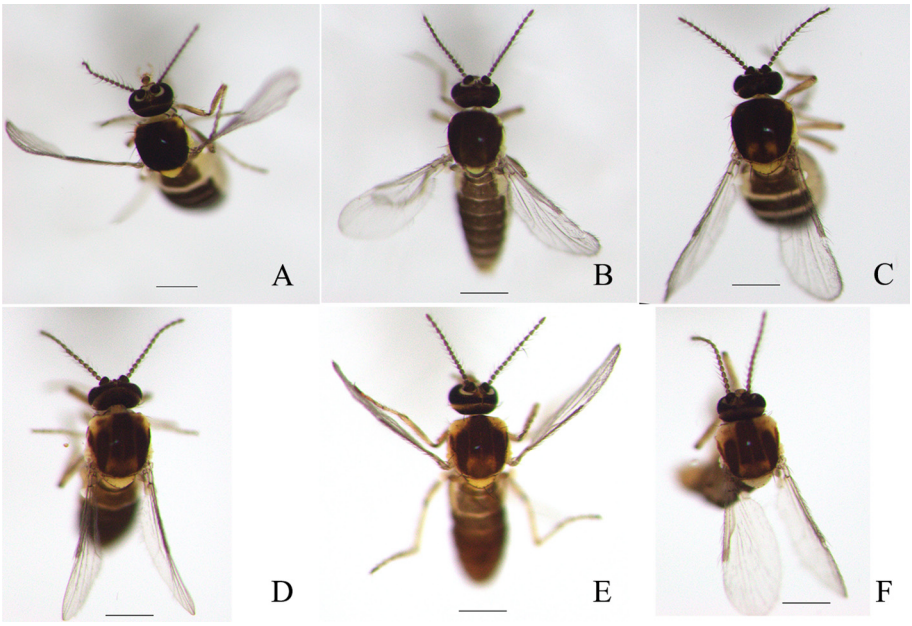

FIGURE 2. Color variation of scutum of *Dasyhelea ludingensis* Zhang & Yu. A. *Dasyhelea ludingensis* W; B. *Dasyhelea ludingensis* type I; C. *Dasyhelea ludingensis* type II; D. *Dasyhelea ludingensis* type III; E. *Dasyhelea ludingensis* type IV; F. *Dasyhelea ludingensis* type V. Scale bar: 500 μm.

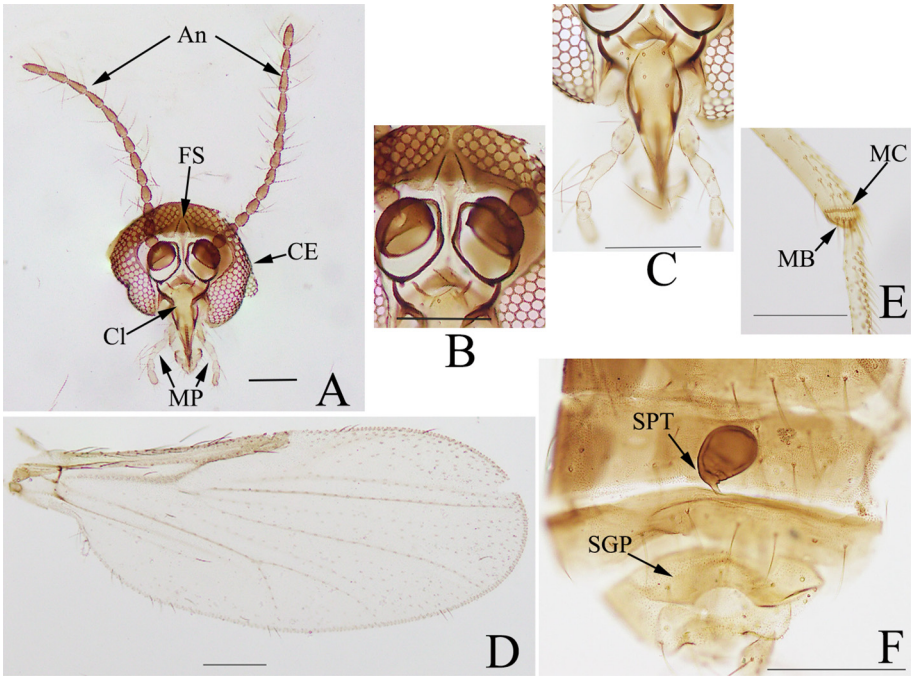

FIGURE 3. Light micrographs of *Dasyhelea ludingensis* Zhang & Yu. A. Head, anterior view; B. Frontal sclerite; C. Palpus and clypeus; D. Wing; E. Hind tibial bristles and combs; F. Abdominal segments 8–10, ventral view. Abbreviations: An antenna; CE compound eye; Cl clypeus; FS frontal sclerite; MP maxillary palps; MB metatibial bristles; MC metatibial combs; SGP subgenital plate; SPT spermathecae. Scale bar: 100 μm.

Thorax. Scutum dark brown uniformly (Figure 2 A) or with yellow lines (Figure 2 B), spots (Figure 2 C) or areas (Figure 2 D–F). Scutellum with 6–7 setae (Figure 4 J). Wing (Figure 3 D) membrane with microtrichia, macrotrichia especially on veins, distal portion and along wing margin; costa extending to about mid–length of wing; 1st radial cell completely obliterated, 2nd radial cell rectangular; wing length 0.69–0.88 (0.79, n = 22) mm, wing width 0.28–0.38 (0.34, n = 22) mm, CR 0.50–0.54 (0.52, n = 22). Legs light brown; tarsi with 5 tarsomeres, claws small, equal-sized, slightly curved inside, (Figure 4 K); hind tibia with 5–7 terminal spines (Figure 3 E, Figure 4 L); prothoracic tarsal ratio 1.77–2.64 (1.99, n = 22); mesothoracic tarsal ratio 2.06–2.40 (2.26, n = 22); metathoracic tarsal ratio 1.97–2.41 (2.12, n = 22).

Abdomen. Brown. One ovoid spermatheca, measuring 27.50–55.00 (45.00, n = 22) μm×22.50–35.00 (30.00, n = 22) μm , with short and twisted neck and the subgenital plate arch-shaped. (Figure 3 F). Cerci ovoid.

Male. Unknown

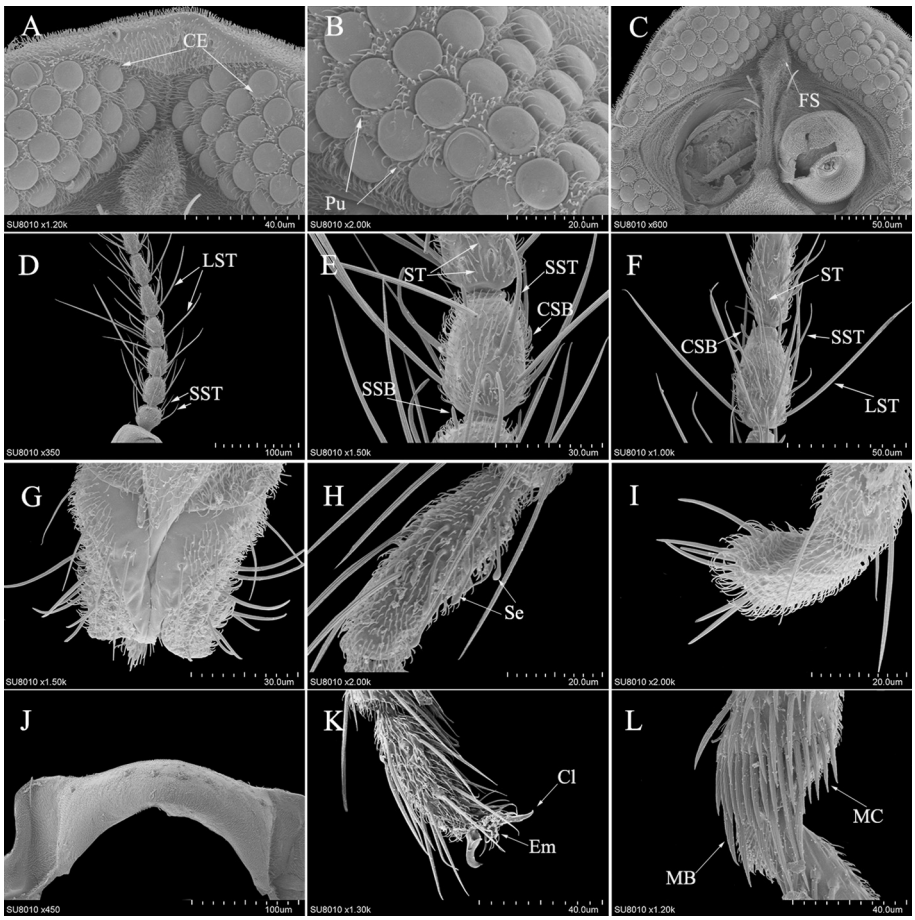

FIGURE 4. Ultrastructure of *Dasyhelea ludingensis* Zhang & Yu. A. Compound eye; B. Ommatidia and pubescences; C. Frontal sclerite; D. Antenna; E. Sensilla of short segments; F. Sensilla of long segments; G. Apex of labrum; H. Segment 3 of maxillary palpus; I. Terminus of maxillary palp; J. Scutellum (dorsal view); K. Tarsomere 5 of hind leg; L. Hind tibial bristles and combs. Abbreviations: CE compound eye; Pu pubescence; FS frontal sclerite; LST long sensilla chaetic; SST short sensilla chaetic; CSB curved sensilla basiconica; ST sensilla trichodea; SSB straight sensilla basiconica; Se sensilla; Em empodium; Cl claw; MB metatibial bristles; MC metatibial combs.

3.2. Distribution

China (Beijing, Shanxi, Shandong, Henan, Shaanxi, Anhui, Jiangsu, Zhejiang, Jiangxi, Hunan, Hubei, Sichuan, Chongqing, Guizhou, Yunnan, Fujian, Taiwan, Guangdong, Guangxi, Hainan, Hong Kong).

3.3. Phylogenetic Analysis

The phylogenetic topology of the genus *Dasyhelea* inferred by the ML method and BI method were shown in Figure 5 and Supplementary Figure S1, respectively. The 15 newly sequenced and the 3 COI sequences from NCBI of *D. ludingensis* were grouped together with high support values in both BI and ML analyses (Figure 5 and Supplementary Figure S1). The similarity among each COI sequence of *D. ludingensis* exceeded 98%, with values ranging from 98.6% to 100% (Supplementary Table S1).

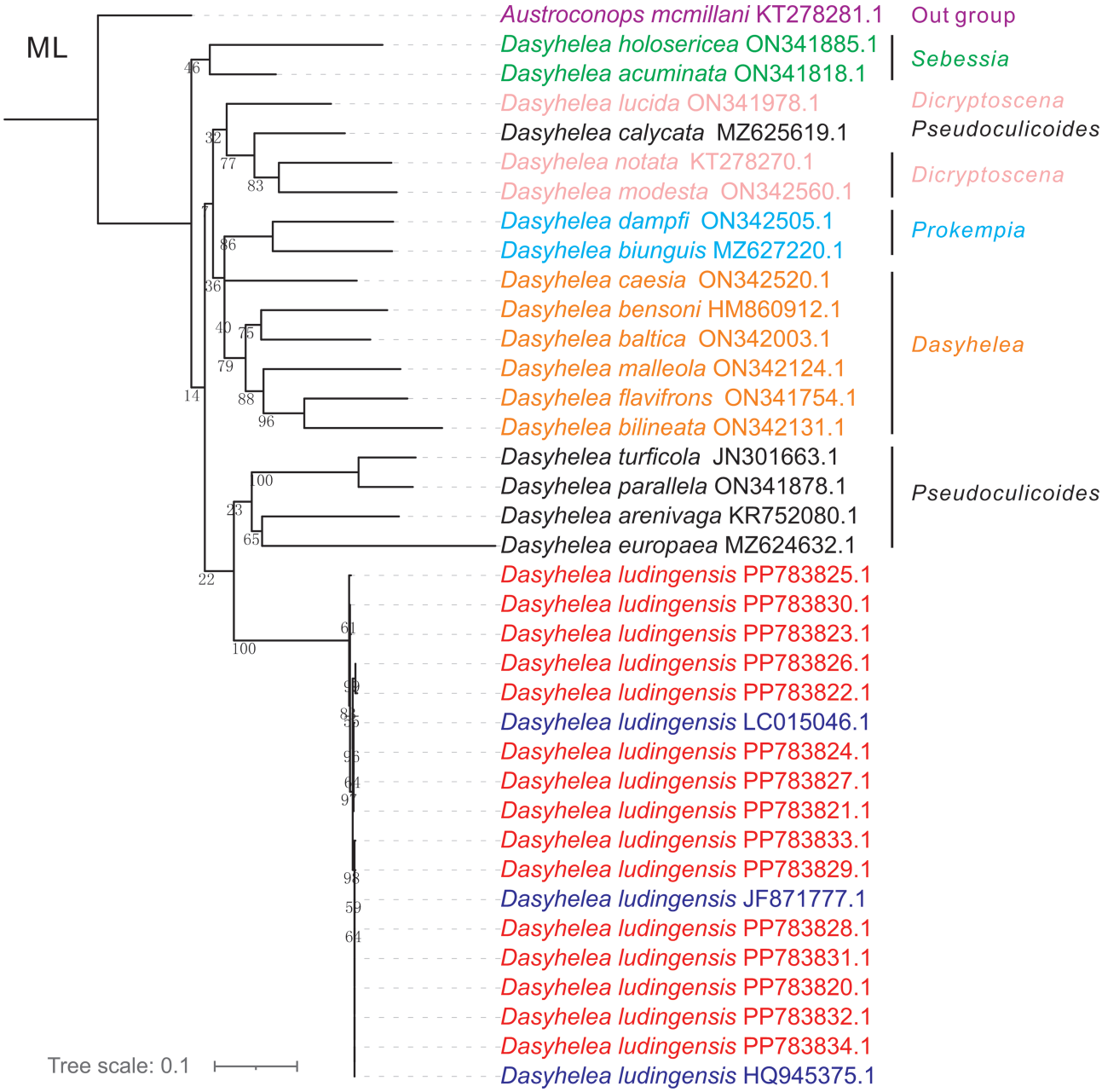

FIGURE 5. The phylogenetic tree was inferred from the nucleotide sequences of COI using IQ-tree via the maximum likelihood (ML) method. The sequences of COI of *D. ludingensis* which marked with blue were downloaded from NCBI, and which marked with red were in this study, respectively.

The topology displayed that *D. ludingensis* was more closely related to species (*Dasyhelea europaea* Remm, *Dasyhelea arenivaga* Macfie, *Dasyhelea parallela* Remm, and *Dasyhelea turficola* Kieffer) belonging to the subgenus *Pseudoculicoides* than species (*Dasyhele dampfi* Kieffer and *Dasyhelea biunguis* Kieffer) belonging to the subgenus *Prokempia* with low support values (Figure 5 and Supplementary Figure S1).

Discussion

Traditional morphological diagnoses of taxonomic status remain widely used (Wattier *et al.* 2020) while an increasing number of studies show that many insect species have intraspecific morphological variation in coloration, size, shape, or other morphological traits. Our present study should be such a case. In this study, we found that the coloring and pattern of coloring in the scutum of *D. ludingensis* female adults in Guizhou geographical population have intraspecific variation. For example, the scutum is dark brown uniformly (Figure 2 A) or with yellow lines (Figure 2 B), spots (Figure 2 C) or areas. Species typing based on the coloring and pattern of coloring in the scutum were carried out. The COI sequences with greater than 98% identity demonstrate that these different types should be classified as the same species. To our knowledge, this is the first report on the intraspecific variation of *D. ludingensis* female adults. The results suggest that the extensive collections and interdisciplinary approaches are necessary for species identification. The more samples collections from different regions and habitats can present the diversity of morphological variation. And the integration of molecular and morphological approach allows assessing the stability and reliability of morphological discriminates for species identification.

When the partial fragment of the *D. ludingensis* COI was cloned by different researchers, multiple pairs of universal primers were available (Folmer *et al*. 1994). Therefore, the COI sequences varied in length. In this study, the ML and BI phylogenetic trees were constructed based on the COI sequences of 658-bp length. A total number of 19 *Dasyhelea* species belonging to 5 subgenera (*Dasyhelea* Kieffer, *Dicryptoscena* Enderlein, *Prokempia* Kieffer, *Pseudoculicoides* Malloch and *Sebessia* Remm) was included. Both ML and BI tree suggested that the *D. ludingensis* was closely associated with the subgenus *Pseudoculicoides* with low support values. *D. ludingensis* was originally assigned to subgenus *Prokempia* by Zhang and Yu (1996), but the result needed to be further confirmed in the future (Zhang and Yu, 1996). More evidence is needed to confirm the subgenera definition of *D. ludingensis* and clarify its phylogenetic relationships. We emphasized the complete mitochondrial genome as a direction for future research.

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Supplementary Materials. The following supporting information can be downloaded at the DOI landing page of this paper.

Supplementary Table S1. The genetic distance between each sequence of 18 COI sequences of *D. ludingensis*.

Additional file 1
Supplementary Figure S1. The phylogenetic tree was inferred from the nucleotide sequences of COI via MrBayes via the Bayesian inference (BI) method. The sequences of COI of *D. ludingensis* which marked with blue were downloaded from NCBI, and which marked with rad were in this study, respectively.

Supplementary Figure S2. Alignment of the nucleotide sequences of 18 COI of the *D. ludingensis*. The identical nucleotide residue was indicated by *. Letters displayed on a yellow background signify sequences that exhibit base differences. The GenBank submission numbers highlighted in red or blue signify that their sequences are identical, respectively.