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Cover: Fish species recorded in the Gowthami-Godavari Estuary, Andhra Pradesh: Lutjanus johnii (top left), Triacanthus biaculeatus (top right), Acentrogobius cyanomos,

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Description of the larva of *Vestalis melania* (Selys, 1873) (Odonata: Calopterygidae) identified through DNA barcoding

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Abstract: The larva of *Vestalis melania* is described and illustrated for the first time, based on specimens collected from Malaybalay, Bukidnon, Philippines. The identity of the larva was confirmed by matching its mitochondrial COI sequence with the adult. The larva can be distinguished by the shape of the prementum and its median cleft, lateral gills, and posterolateral abdominal spines. Comparison with other known larvae in the genus is also provided. The significance of using DNA barcoding for identifying larvae of Philippine Odonata is emphasized.

Keywords: COI sequencing, Odonata, Philippines, Mindanao, Zygoptera

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Author contributions: DMEG—conceptualization, data curation, methodology, investigation, formal analysis, writing; OMN: supervision, methodology, writing; SRRT—supervision, methodology, investigation, validation, writing; RJTV—supervision, validation, writing.

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INTRODUCTION

Vestalis Selys, 1853 is a genus of the Calopterygidae family with 16 species distributed in the Oriental region (Lieftinck 1965; Paulson & Schorr 2021). Like other members of Calopterygidae, the species thrive well in pristine habitats with good water quality (Orr 2003). In the past, the genus was subdivided into three groups, which were treated as full genera based on neural and penile characters (May 1935). These three are *Vestalis* Selys, 1853, *Vestinus* Kennedy, 1920, and *Vestalaria* May, 1953. Lieftinck (1965) dismissed this division, stating the instability of the characters defining *Vestinus*. However, molecular and morphological data supported the resurrection of the genus name *Vestalaria* (Hämäläinen 2006).

Vestalis melania, a member of the genus Vestalis, is geographically distinct for its insular distribution and restriction in the Philippines (Lieftinck 1965). The species is widely distributed in the country, except in Palawan, thrives mainly in the open or partly shaded streams and rivers (Villanueva 2009). Presently, only two of the 16 species within Vestalis have described larvae which are V. amoena and V. luctuosa (Ris 1912; Lieftinck 1965). Hence, in this study, the larva of V. melania was described for the first time. Larval identity was confirmed by matching the mitochondrial COI sequence of larvae and adults, a method increasingly utilized in Odonata (Orr & Dow 2015a,b, 2016; Steinhoff et al. 2016; Yu 2016; Wang et al. 2017; Saetung & Boonsoong 2019). Detailed morphology of the larva was also described and compared with other known larvae in the genus to gain more insights into its phylogenetic position.

MATERIALS AND METHODS

Collection of Specimens

Larval specimens were collected from the streams of Kibalabag, Malaybalay City, and Bukidnon. Specimens were collected through sieving substrates, leaf debris, and water vegetation in the margins of streams or water pockets near streams. Samples collected were preserved in 95% ethanol. All materials are deposited in the Natural Science Museum (NSM-4293 to NSM-4296) of Mindanao State University-Iligan Institute of Technology, Iligan City, Mindanao, Philippines. The collection was made under the DENR wildlife gratuitous permit no. R10-2021-27.

DNA Extraction and Polymerase Chain Reaction

Genomic DNA was extracted from the legs of

specimens using the EZ-10 Spin Column Genomic DNA Minipreps Kit (BioBasic, Canada). The animal DNA barcode, COI (cytochrome c oxidase subunit I), was amplified by universal primers (5'GCTCAACAAATCATAAAGAYATYGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAARAAYTCA-3') (Folmer et al. 1994). Each PCR reaction contains 30 µL of PCR master mix (Bio Basic, Inc.), 18 µL of ddH2O, 3 µL of each primer, and 6 μ L of DNA template for a total volume of 60 μ L. The PCR thermal regime consisted of pre-denaturation at 94 °C for four mins; 35 cycles of denaturation at 94°C for 30 sec., annealing at 48.5 °C for 30 sec., and extension at 72 °C for 90 sec.; final extension at 72 °C for seven mins; and hold for 4 °C at ∞. PCR products were then subsequently visualized on 1.5% agarose gel (Biorad) using blueGel electrophoresis system (Minipcrbio, Amyplus). PCR products were then sent to Macrogen Korea for sequencing.

DNA Barcode Analysis

The forward and reverse COI sequences were edited using Snapgene Viewer 5.2.5.1 (GSL Biotech; available at snapgene.com). Consensus sequences were then generated through queries of the forward and reverse sequence in NCBI Blast. Sequence analyses were carried out using MEGA 10 (Kumar et al. 2018). Pairwise distances were calculated using Kimura-2-parameter model using all sites and 1,000 bootstrap replications to determine the genetic distance between conspecific individuals.

Imaging and Description

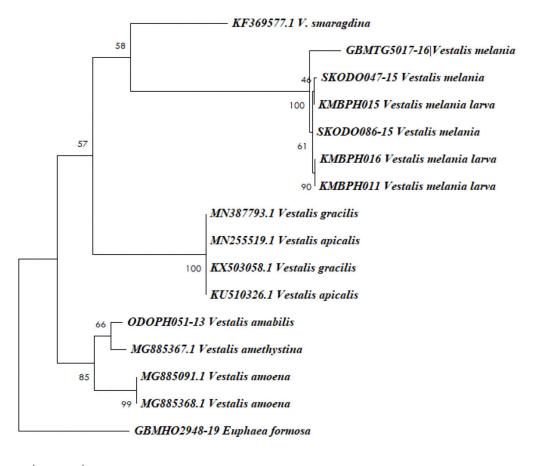
Specimens were examined and photographed using a stereo microscope with an attached digital camera (AmScope) and a Canon EOS 60d. Illustrations were created through an Ipad using the procreate application (Savage Interactive, Australia), based on representative images. Measurements were obtained through ImageJ (Schneider et al. 2012). Terminologies for the larval morphology were based on Snodgrass (1954) and Kumar (1973). The mandibular formula follows Watson (1955). Abdominal segments 1–10 were indicated as S1–S10.

RESULTS

The COI sequences of all samples were amplified and sequenced successfully, producing barcodes 568– 576 bp long. A maximum-likelihood tree including 11 reference sequences from *Vestalis* and *Vestalaria* (Table 1) is shown in Figure 1. *Euaphaea formosa* was used as

Tab	le 1. 9	Specimen	data of	COI	sequence	used i	n th	ne ana	lysis.
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Species	Data Source	ID/AN	Locality	Date	Collector
Vestalis melania	BOLD	SKODO086-15	Tagbina, Surigao del Sur, Philippines	27.ii.2015	H. Cahilog
Vestalis melania	BOLD	SKODO047-15	Tboli, South Cotabato, Philippines	22.viii.2015	H. Cahilog
Vestalis melania*	This study	KMBPH011/ NSM-4293	Kibalabag, Malaybalay City, Philippines	14.x.2020	D.M. Guadalquiver
Vestalis melania*	This study	KMBPH015/ NSM-4294	Kibalabag, Malaybalay City, Philippines	14.x.2020	D.M. Guadalquiver
Vestalis melania *	This study	KMBP016/ NSM-4295	Kibalabag, Malaybalay City, Philippines	14.x.2020	D.M. Guadalquiver
Vestalis amethystina	NCBI	MG885367.1	Singapore		
Vestalis amabilis	NCBI	KF369567.1	Sarawak, Malaysia	01.01.2010	J. Teo
Vestalis amoena	NCBI	MG885091	Singapore		
Vestalis amoena	NCBI	MG885368.1	Singapore		
Vestalis apicalis	NCBI	KU510326.1	India		
Vestalis apicalis	NCBI	MN255519.1	India		
Vestalis gracilis	NCBI	KX503058	India		
Vestalis gracilis	NCBI	MN387793.1	India		
Vestalis smaragdina	NCBI	KF369577.1			
Euphaea formosa	BOLD	GBMHO2948-19	Taiwan		



0.10

Figure 1. Phylogenetic reconstruction based on COI (587 bp), through maximum likelihood method and 1,000 bootstrap replication. Bootstrap values are indicated at nodes respectively. *Euphae formosa* was used as an outgroup.



Image 1. Larval habitus of *Vestalis melania*. Scale = 5 mm. © Don Mark E. Guadalquiver.

an outgroup. The adults of *Vestalis melania* distinctly formed a monophyletic group (MLB = 100) with prospect larvae. *Vestalis smaragdina*, a member of *Vestalaria*, also had the closest relationship with *V. melania*, forming a monophylum (MLB = 58). The results confirmed that the larvae concerned here are of the same species as adults, namely *V. melania*.

Taxonomic Account

Vestalis melania Selys, 1873

Materials studied: Larvae: 14.x.2020, 1 male, 3 females: Kibalabag, Malaybalay City, Bukidnon, Philippines (8.258 N, 125.172 E), 1,200 m, coll. D.M. Guadalquiver, Natural Science Museum, MSU-IIT, Iligan, Philippines

Description: A slender zygopteran with a small head, moderately long antennae, laterally banded thorax with long and banded legs, elongated and cylindrical abdomen with lanceolate lamellae. Ground color of light brown but can be darker in some individuals (Image 1).

Head: Hexagon-shaped with a pointed snout, flattened above, with light banding, pointed & pigmented postocular lobe, and eyes longer than wide when dorsally viewed. Antennae (Figure 2a) seven-segmented excluding extra joint after segment 1, tapered from base to apex, with robust segment one almost twice as long as segments 2-7. Prementum (Figure 2b) elongated with the distal end expanding at angles 110 to 125° wide. Median lobes (Ligula) clefted roundly and with deepness 0.36 of the prementum, serrated on the outside, and containing a pair of setae. Labial palp robust, the inner lateral margin serrated with two sizes of teeth, and with three strong, long, and incurved distal teeth, of which the middle one is the longest; movable hook very long and robust with two setae on its base. Maxilla (Figure 2c) is twice as long as wide; galeo-laccinia with seven teeth: four long in the dorsal area and three short in the ventral area, and with numerous hair-like projections.

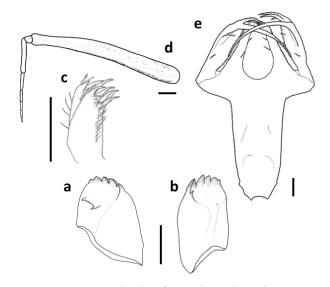
Palpus is two-segmented, with a small basal segment and distal segment that is banana-shaped but pointed, as long as galeo-laccinia, and covered in numerous hair-like projections. Mandibles (Figure 2d,e) with the formula L 1'1234 0 a($m^{1,2,3,4,5-7}$)b/ R 1'1234 y a. Left mandible with five incisors and molar crest with 5–7 fine cusps; right mandible with five incisors, an extra tooth, and a single mandible.

Thorax: Marked with strong bandings in the lateral area extending from the pronotum up to the dorsal region of synthorax. Prothorax smaller than head and synthorax. Pronotum hexagonal with a protuberance at the mediolateral proximities. Wing pads reaching the proximal margin of S4. Legs long and with two dark bands in femur and tibia and progressively longer from pro- to meta-thorax. Tibia longer than femur; tarsi three-segmented and covered with dense hair.

Abdomen: Long & slender and covered with dark pigmentation, amount varying between specimens, but less pigmented on the median region. Lateral spines on S9 and S10, with S10 spine more prominent (Figure 3a,b).

Table 2. Comparison of	f Characters f	rom Vestalis and	Vestalaria	larvae.
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	V. melania	V. luctuosa	V. amoena	V. venusta
Premental cleft	Round	Angled	Angled, narrow	Round
Anterior region of prementum	With angular expansion (110–125° angle)	Expanding obliquely	With angular expansion (110–125° angle)	Expanding obliquely
Premental setae	Two pairs	Two pairs	Two pairs	Three pairs
Lateral gills	Lamellate, longer than the middle, with light/ unpigmented spots in the edge	Lamellate, with dark spots in the edge, slim	Lamellate, truncated, almost similar length with middle gill, stout	Triquetral, significantly longer than the middle, slim
Vestigial joint between segments 1 and 2 of antenna	Present	Present	Present	Absent
Posterolateral spines in abdomen	S9–10, with S10 very prominent	S10, prominent	-	S10, inconspicuous



Male gonapophyses protruding from the middle of S9, small and conical with black pigmentations in the upper lateral area (Figure 3a). Female inner gonapophyses large and extending from proximal margin of S9 to distal margin of S10; outer part protruding from middle of S9 to distal margin of S10, with distal region slight pointed upward (Figure 3b). Male cerci small and budlike; female cerci more pointed and slightly longer than male ones. Caudal gills are all lamellate, long, lanceolate shaped but blunt-tipped, and bearing some fine setae-like spines along margins. The lateral caudal gill (Figure 3d) is longer than the middle gill, with a prominent midrib and light to dark pigmentation covering the entire median region; banding manifests only in the lateral edges. Middle caudal gill (Figure 3c) with full banded pigmentation, translucent, and visible median venations.

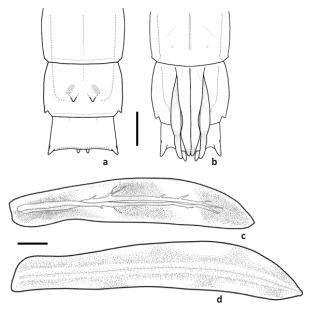


Figure 3. Posterior details of Vestalis melania larvae: a—S8–S10 of abdomen showing male gonopophyses, lateral spines, and cerci | b—S8–S10 of abdomen showing female gonopophyses, lateral spines, and cerci | c—median caudal gills, lateral view | d—lateral caudal gills, lateral view. Scale = 1 mm

Microhabitat and Behavior

Larvae were found in an unshaded, narrow, montane stream with a sandy substrate and dense marginal and submerged vegetation (Image 2). Larvae are abundant where they are found and were found clinging and scooped along with submerged vegetation. Adults of *V. melania* and *Euphaea amphicyana* were also abundant in the area.

DISCUSSION

The only described species within the genus Vestalis are V. luctuosa (Ris 1912; Lieftinck 1965) and V. amoena (Lieftinck 1965). Comparison of the larval morphology of V. melania from descriptions of these species shows that V. melania is different in several aspects. Vestalis melania shows stronger banding in the pronotum up to the thorax; round median cleft in the prementum compared to angular base, and sharp broadening in the anterior region of the prementum compared with gradual broadening in the other species.

The lateral gills also differ with *V. luctuosa* and *V. amoena*, in terms of pigmentation. In *V. melania*, pigmentation was concentrated and full in the central region, and the banding is observable only in the edges, giving it an appearance of having 'white' spots in the borders. In contrast, the lateral gills of *V. luctuosa* are less pigmented and show dark spots in the edges (Ris 1912), whereas *V. amoena* does not show much pigmentation and has a truncated shape (Lieftinck 1965) (Table 2).

The posterolateral spines in the abdomen of V.

melania were also remarkable, being prominent in S9– 10. In *V. amoena* and *V. luctuosa* (Lieftinck 1965), a small spine is also present in S10, but it is unclear if it is also present in S9.

Overall, the larval characteristics of *V. melania* are different in terms of stronger banding in the pronotum and thorax, characters in the prementum, lateral caudal gill, and posterolateral spines in the abdomen.

This study demonstrates once again the usefulness of DNA barcoding in matching the larvae with the adult. This method can be utilized to gain larval knowledge of endemic Philippine Odonata, especially endemic genus like *Risiocnemis*. As the marker COI has been proven helpful in differentiating many Philippine damselfly species (Casas et al. 2018), it can be effectively utilized to match most larvae and adults of the same species. Caution, however, should be observed in using COI genes for some species groups, as the gene may not be well-differentiated in some closely related species such as Philippine *Drepanosticta* species (Casas et al. 2018). Another example is the *Vestalis gracilis* and *V. apicalis* used in this study which showed no divergence (MLB



Image 2. Habitat of Vestalis melania larvae in Kibalabag, Malaybalay City, Bukidnon, Philippines. © Don Mark E. Guadalquiver.

= 100). Hence, other gene targets should be utilized as well, such as the nuclear ribosomal genes and the internal transcribed spacers.

CONCLUSION

This study describes for the first time the larvae of Vestalis melania. The mitochondrial COI sequence successfully matched the V. melania larvae with its adults and confirmed its identity, in congruence with their sympatric relationship. The larva of V. melania is highly similar to previously described congener species but different in terms of stronger banding in the pronotum and thorax, characters in the prementum, lateral caudal gill, and posterolateral spines in the abdomen. The larval morphology of the V. melania supports the unity within the genus Vestalis and the separate genus status of Vestalaria. It is recommended that larvae of other Vestalis species be further studied and DNA barcoding, should be incorporated to gain more larval knowledge of endemic Philippine Odonata. Because of the limitations of the COI marker in closely related species, it is also recommended that other gene targets and relevant data should be used for support.

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