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Description of a new Oriental stonefly species, *Phanoperla constanspina* (Plecoptera: Perlidae) from Mindanao, Philippines and association of life stages using DNA barcoding

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Abstract

A new perlid stonefly, *Phanoperla constanspina* **sp. nov**. is described from Mt. Malindang, northern Mindanao, Philippines. The male of the new species is distinguished by lacking lobes on penial sac and large black spines at the penial apex. The female is distinguished by the egg. DNA barcoding was used to associate male, female, and nymphal specimens with 0% divergence. Morphological variation was observed in the shape of the hemitergal anterior processes and the 9th tergal setal patches of male adult and body pigmentation of the nymph. A key to the Philippine *Phanoperla* species and a checklist of Oriental *Phanoperla* are also provided.

Key words: DNA barcoding, Egg morphology, Mindanao, Mt. Malindang, *Phanoperla*, Philippine archipelago

Introduction

Phanoperla is an Oriental genus distributed west of the Indian Himalayas and Sri Lanka (Zwick, 1982; Stark, 1983) of mainland Indochina (Zwick, 1982; Stark, 1983; Zwick & Sivec, 1985; Bae *et al*, 2009; Sivec & Stark, 2010) and extending eastward into the archipelagic islands of Sumatra (Zwick, 1982; Zwick & Sivec, 1985), Java (Stark & Sivec, 2007), Borneo (Zwick, 1982; Stark & Sivec, 2007; Stark & Sheldon, 2009) and the Philippines (Zwick, 1982; Sivec & Stark, 2011). *Phanoperla* species are generally widespread throughout the Oriental Region (Zwick, 1982), but a few species appear to be endemic such as *P. himalayana* Zwick 1977 distributed narrowly in the Himalayans and *P. testacea* Zwick 1982 only known from Sri Lanka.

The number of *Phanoperla* Banks 1938 species has increased from 35 species in the 1980's to currently 51 species as a result of discoveries of new taxa from Thailand, Vietnam, Borneo, and the Philippine Islands. However, only seven species of *Phanoperla* have been described from the Philippine Archipelago. One Philippine *Phanoperla* species, *P. claggi* Banks 1938 is considered a *nomen nudum* (Zwick 1982). Zwick (1982) indicated that *P. claggi* was not listed by Claassen (1940), Illies (1966), and Zwick (1973). Among Philippine *Phanoperla*, *P. flaveola* Klapalek (1910) and *P. bakeri* Banks (1924) are also known from Borneo. Morphologically, *P. omega* Zwick (1982) is distinguished from *P. flaveola* and *P. bakeri* by a three-branched Rs (radial sector) of the forewing instead of two-branched Rs. Recently, Sivec and Stark (2011) described three additional new *Phanoperla* species collected in 1966 from the Palawan Island, Philippines (*P. batac*, *P. circumspina*, *P. magnaspina*). Sivec & Stark (2011) utilized adult characters, including tergal tips and everted penial sacs of males and vaginal sacs and eggs of females. The nymphal stage of the genus is still poorly understood. Zwick (1982) distinguished *Phanoperla* from the hyper diverse stonefly genus, *Neoperla* Needham 1905 using characters of penial sac of males. Zwick (1982) indicated that *Phanoperla* is still in need of study and identifications are often problematic. For example, the large hooks of the penial sacs of *Phanoperla* have been mistakenly identified as sclerotized plates, which are also frequently observed in *Neoperla*. Zwick (1982) classified the Philippine *Phanoperla* into seven species complexes: *anomala*, *maindroni*, *nana*, *pallipennis*, *flaveola*, *nervosa* and *testacea*, based mainly on the structure of male

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everted penial sacs (Zwick, 1982). Additionally, three species of Philippine *Phanoperla* were considered as *incertae sedis* and several unnamed species were based on female adults and nymphs (Zwick, 1982).

The utility of DNA barcoding has been a prominent tool in the identification and association of several organisms (Blaxter, 2004). The Barcoding of Life Project (Hebert *et al*., 2003a,b) initiated a standardized method to improve species identification wherein the short DNA sequences from a standardized region of the genome provide DNA barcodes on one target gene, cytochrome *c* oxidase subunit 1 *(cox1)* (Hebert *et al*, 2004a). According to studies of Hebert (2003a; 2004a,b), *cox1* sequence clustering by neighbor joining has been suggested as an effective and suitable way to recognize and associate animal species as well as discover cryptic taxa. DNA barcoding has the possibility of associating different life stages (Blaxter, 2004; Stoeckle, 2003). This is important when taxa are difficult to rear in laboratory, and several studies have recognized the benefit of DNA sequences in associating immature stages with adult counterparts (Hebert *et al*, 2004b; Barrett & Hebert, 2005; Paquin & Hedin, 2004; Thomas *et al* 2005). Also, many of aquatic insects are particularly challenging because of the taxonomic keys only often exist for adult males (Ekrem *et al*, 2007, Ball *et al* 2005). DNA barcoding has previously been used to identify distinct mayfly species (Ephemeroptera) (Ball *et al* 2005; Alexander *et al* 2009), validate morphological taxonomy of stoneflies (Plecoptera) (Boumans & Baumann, 2012; Heinold *et al* 2014), and associate larval and adult caddisflies (Trichoptera) (Zhou *et al* 2007; Waringer *et al* 2008; Paul *et al* 2010).

In this study, we describe a new *Phanoperla*, *P. constanspina* **sp. nov**. from Mt. Malindang on Mindanao Island, Philippines based on the structure of everted penial sac of males, egg morphology, and nymphal morphology. The female and the nymph of *P. constanspina* **sp. nov**. were associated using DNA barcodes. We also provide a key for identifying males of seven valid *Phanoperla* species currently recognized from the Philippines based on male genitalic characters (Zwick, 1982, Sivec & Stark, 2011) and a checklist of the known species from Oriental Region.

Material and methods

Adults and nymphs were collected along the riparian area of Layawan River in Mt. Malindang, Mindanao Island, Philippines (N 08°18. 495' E 123°37.980', 1,218 masl; N 08°19.417' E 123°38.017', 956 masl; N 08°21.848' E 123°38.227', 610 masl; N 08°23.690' E 123°39.116', 460 masl; N 08°23.690' E 123°40.056', 336 masl; N 08°24.452' E 123°40.887', 264 masl; N 08°25.418' E 123°41.806', 185 masl; N 08°25.971' E 123°42.492', 161 masl; N 08°26.314' E 123°42.492', 127 masl; N 08°27.290' E 123°44.697', 73 masl). Adults were obtained using light traps and nymphs were collected from under cobbles and from dead leaves in the river. All individuals were preserved in Eppendorf tubes with 95% ethanol and later stored at -20°C. A stereo microscope (Olympus SZ51 & SZ61) with an attached digital camera (Canon EOS700) was used to examine and image specimens under magnification of approximately 0.67-4.5x. Images were prepared in Adobe Illustrator CS6 (v.16.0.0, Adobe Systems Inc.). Measurements of the individuals were obtained using ImageJ (v.1.48). The aedeagi of males were dissected and treated with 4°C 15% KOH (Zwick, 1983) until the structures appear to be clear with visible inner sacs. Penial sacs were partially everted for a detailed view of the structure because of their small capsule sizes and fragile membranes. Eggs were retrieved from dissected 8th sternite of females and examined with using a scanning electron microscope (SEM) (Hitachi, SU1510).

We extracted genomic DNA from the insect legs following a standard protocol outlined in Lin & Wood (2002). The animal DNA barcode *CO1* (*cytochrome c oxidase subunit I*) was amplified using the primers, LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al*, 1994). Each Taq reaction buffer contained 5 µL of TAE buffer, 35 µL of ddH_2O, 4 µL dNTPs, 1 unit of Taq polymerase, 2 µL of each primer, and 1 µL DNA template. The PCR thermal regime consisted of 35 cycles of 60 sec of denaturing at 94°C, 45 sec of annealing at 53°C, 60 sec of extension at 72°C and then 10 minutes at 72°C. PCR products were subsequently visualized and purified on 1.5% agarose gel. DNA sequencing was carried out using the BigDye® terminator 3.1 sequencing kit on an ABI 3730XL DNA Analyzer (Applied Biosystems). The sequences were edited and assembled manually using SeqMan Pro (v.7.1.0, DNASTAR Lasergene), and then aligned using Clustal W in MegAlign (v.7.1.0, DNASTAR Lasergene). Pairwise distances between sequences were calculated using Kimura-2-Parameter (K2P) model of nucleotide substitution (Table 4). DNA sequences were deposited in GenBank (Table 1).

TABLE 1. GenBank accession numbers of *Phanoperla constanspina* **sp. nov**. (Plecoptera: Perlidae) specimens used for DNA barcoding.

Taxon	Haplotype Code	Sex	Life Stage	Accession Number
Phanoperla constanspina sp. nov.	A-G23.m	♂	Adult	KT353028
Phanoperla constanspina sp. nov.	A-H7.m	♂	Adult	KT353025
Phanoperla constanspina sp. nov.	A-H16.m	♂	Adult	KT353024
Phanoperla constanspina sp. nov.	A-H12.m	♂	Adult	KT353026
Phanoperla constanspina sp. nov.	A-E23.m	♂	Adult	KT353027
Phanoperla constanspina sp. nov.	A-H11.m	♂	Adult	KT353023
Phanoperla constanspina sp. nov.	A-G11.f	♀	Adult	KT353022
Phanoperla constanspina sp. nov.	N-J25.m	♂	Nymph	KT353021
Phanoperla constanspina sp. nov.	N-G49.m	♂	Nymph	KT353019
Phanoperla constanspina sp. nov.	N-H20.f	♀	Nymph	KT353018

Results

Phanoperla constanspina sp. nov. dela Cruz, Nuñeza, and Lin

(Figs. 1–11)

Material examined: Philippine Islands, Mindanao, Mt. Malindang, Layawan River. Holotype male, 185 masl, N 08°25.418' E 123°41.806', 3 October 2013 (A-G23.m). Paratypes 5 males: 1 male, 336 masl, N 08°23.690' E 123°40.056', 1 October, 2013 (A-E23.m); 4 males, 161 masl, N 08°25.971' E 123°42.492', 4 October 2013 (A-H7.m, A-H11.m, A-H12.m, A-H16.m). Nymphal paratypes 3 males: 185 masl, N 08°25.418' E 123°41.806', 3 October, 2013 (N-G49.m); 161 masl, N 08°25.971' E 123°42.492', 4 October, 2013 (N-H27.m); 73 masl, N 08°27.290' E 123°44.697', 6 October, 2013 (N-J26.m). Nymphal paratypes: 2 males, 3 females, 956 masl, N 08°19.417' E 123°38.017', 16 April 2014 (N-B30.f); 1 male at 264 masl, N 08°24.452' E 123°40.887', 2 October 2013 (N-F20.f). The Holotype is deposited in the collection of the Natural Science Museum, MSU-IIT, Philippines and paratypes in the Insect Collection, National Museum of Natural Science, Taichung, Taiwan (NMNS). All specimens were collected and prepared by INDC.

Adult habitus. General color brown, sometimes appearing dark brown when membranous dark wings fold flat at rest. Head wider than pronotum, bearing a distinctive stretched M-shaped pattern, appearing to be paler to surrounding area (Figs. 1A and 1B). Frons with dark brown pigment along occipital line and ridge (Fig. 1A). Compound eyes bulging, relatively large. Ocelli of 0.76 ± 0.1 mm in diameter in males and 1.0 ± 0.0 mm diameter in females, with lateral inner dark rings. Median occipital line conspicuous and well-marked. Median suture forms a bare depression with raised margins. Antennae and palpi pale brown basally, distally dark. Pronotum rectangular with rounded corners, dark lines occur on margins and median suture, rugosities conspicuous on pronotum. Smaller hairs cover pronotum and most of the head. Wings brown and slightly infuscated with dark-venation (Figs. 2A and 2B). Legs dark brown from the proximal half of femora towards tibia and tarsus (Fig. 3) . Small thorn-like hairs present particularly on femoral margins.

Male. Body length 9.12 ± 0.51 mm, forewing 9.35 ± 0.47 mm, hindwing 8.25 ± 0.2 mm. Width of head at pronotum 1.62 ± 0.13/1.5 ± 0.1 mm (Fig. 1A). Femur 1.91 ± 0.03 mm, tibia 2.28 ± 0.06 mm, tarsus 0.62 ± 0.03 mm (Fig. 3). Brushes of long setae on abdominal sternites S4-S8, usually strongest on S5-S7 (Fig. 4). Tergites simple (T7 and T8), T9 with a median depression but slightly raised at sides with short conical-shaped setae (Fig. 5). Anterior process of the hemitergite unbranched and of variable shape (Fig. 5). Penial capsule short, 0.76 ± 0.1 mm, but tubular at base, faintly sclerotized for muscle attachment (Fig. 6). Everted sac almost equal to penial tube in length and armed with small jet-black posterior spines, fewer and becoming shortest towards the base (Fig. 6). Apex of the sac cylindrical and black covered with short and fine black hair-like spines of uniform length on anterior surrounding structure (Fig. 6).

Female. Body length 12.6 mm, forewing 12.46 mm, hindwing 12.1 mm. Head/pronotum width 2.23/2.09 mm

(Fig. 2A). Femur 2.06 mm, tibia 2.69 mm, tarsus 0.85 mm. Subgenital plate undeveloped, displaying a simple posterior margin on S8.

Egg. General outline droplet-shaped, somewhat elongated, and very similar to *P. batac* (Fig. 8A). Total length ca. 522.98 ± 0.06 µm and equatorial width ca. 198.6 ± 0.12 µm, with long and slender collar with a length of 137.75 ± 0.23 µm. Rugose processes are observed on the collar extension while the apex is flanged with a cork-like rim (Fig. 8C). Chorionic surface even and simple but with few, fine punctations (Fig. 8B) around the subapical ring of the anterior end (Fig. 8D).

FIGURES 1. *Phanoperla constanspina* **sp. nov**. head and pronotum. (1A) Male adult, indicating occipital line. (1B) Female adult. Scale = 1.00 mm.

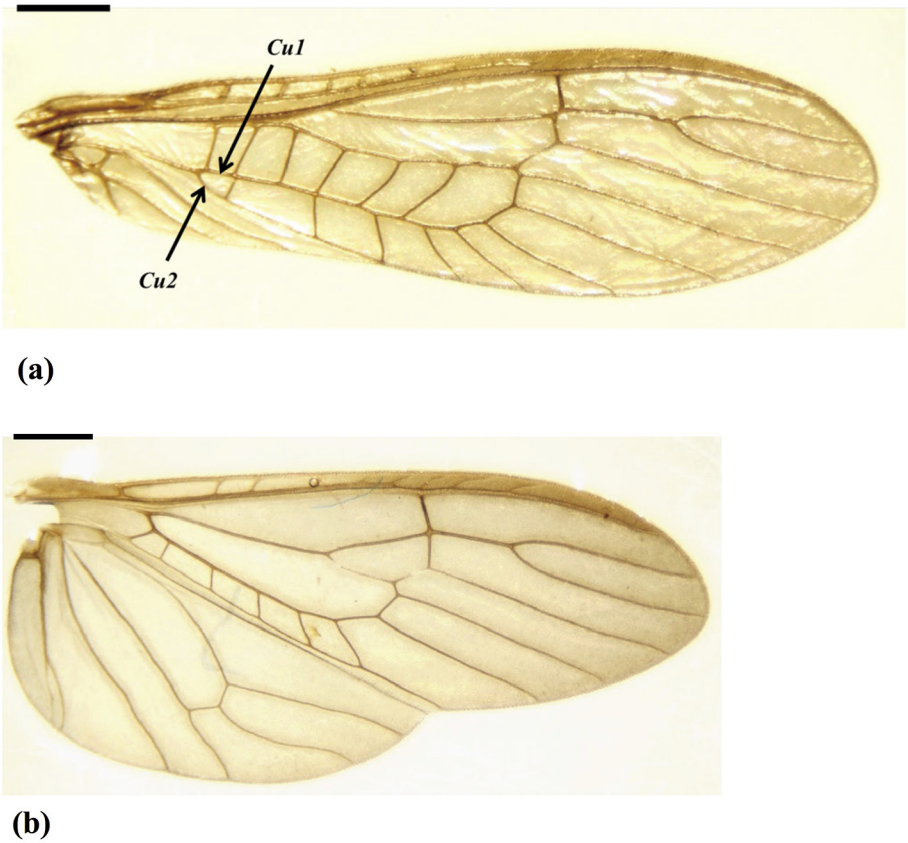
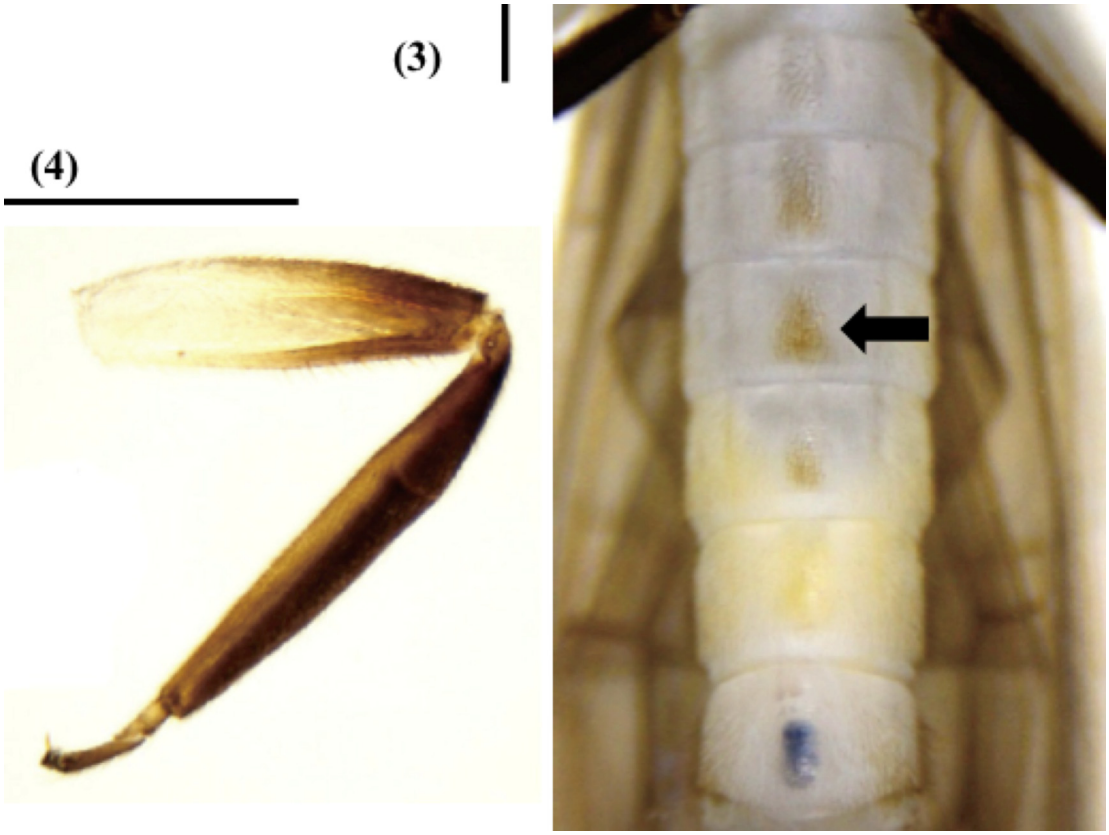


FIGURE 2. *Phanoperla constanspina* **sp. nov**. male wing. (2A) Right forewing, showing the forking of Cu_2 from Cu_1. (2B) Right hindwing. Scale = 1.00 mm.



FIGURES 3–4. *Phanoperla constanspina* **sp. nov**. male. (3) Right foreleg. (4) Abdominal sternite showing hair brushes on each segment (S5–S8). Penial capsule visible on S9. Scale = 1.00 mm.

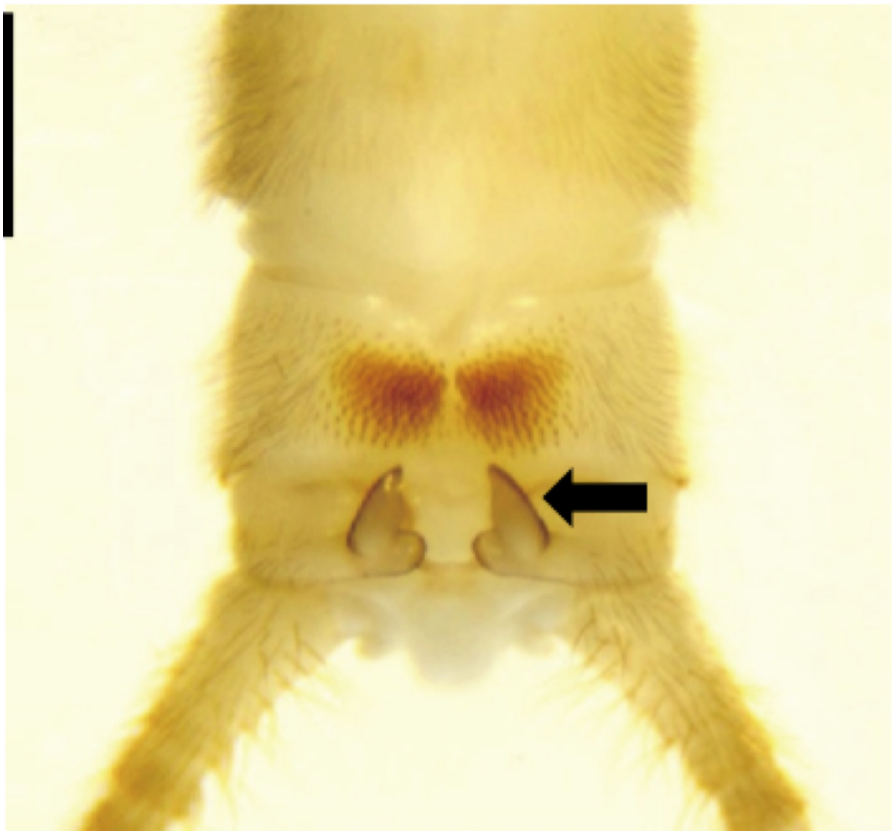


FIGURE 5. *Phanoperla constanspina* **sp. nov**. male dorsal terminalia showing the hemitergal (arrow) and paired clumps of short setae located on medial tergum 9. Scale = 1.00 mm.

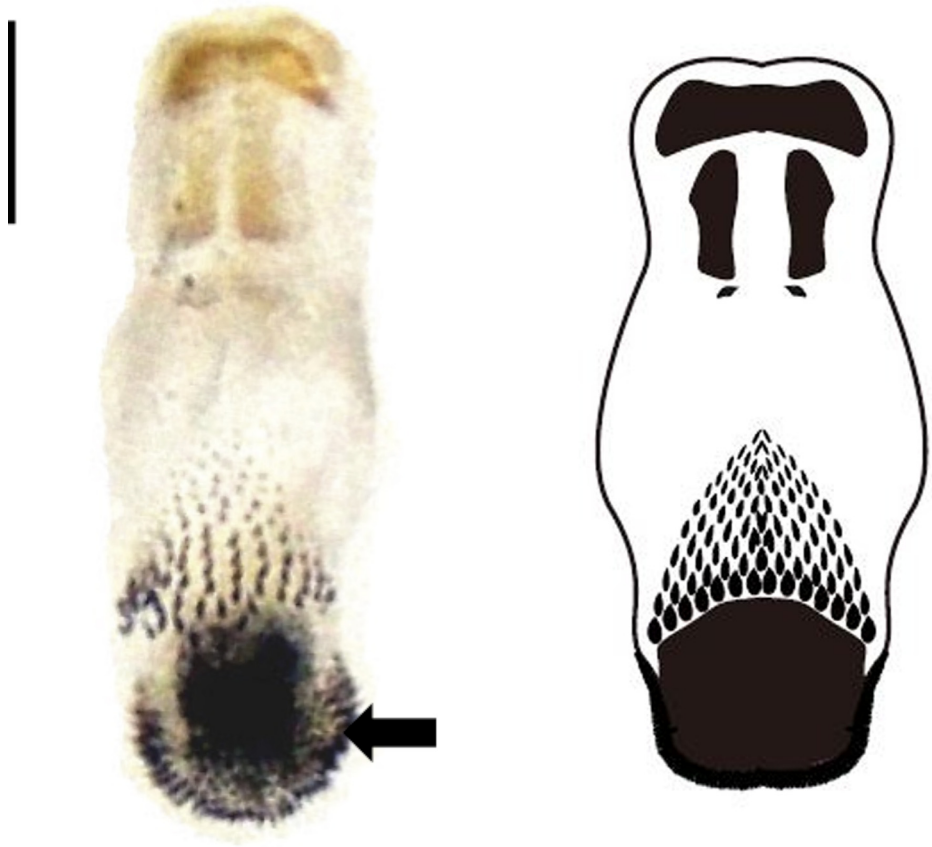


FIGURE 6. *Phanoperla constanspina* **sp. nov**. penial tube showing the everted penial sac at the base. Scale = 0.2mm.

Nymphal habitus. Females are considerably larger than males. General pigment pattern variable in mature individuals, ranging from light to dark brown and sometimes black with varying hues and pigment intensity on a few structures such as tips of wingpad and tergal margins (Figs. 11A and 11B). Heads always with darker frons and paler M-line pattern, which is evident on the adults. Well-marked horizontal suture separating labrum from head, making a protruding labrum slightly noticeable. Pronotal disc usually wider than head, occipital ridge positioned more forward from posterior margin of head base (Figs. 11A and 11B). Pronotum oval-shaped. Wingpads medium and simple. Abdomen slender relative to body size with obvious stripes separating terga from each other (Figs. 11A and 11B). Antennal flagellum long and setaceous, cercus medium-sized and bearing short, stiff hairs. Basal attachment of cerci is a little plump extending wide from the base of T10 while the apical tip becomes thin. Mandible ovate, base plumper than the rest of the structure (Fig. 9). Teeth uneven in length and size, slightly bent towards the inner side, brushes of bristled hair present laterally and adjacent to the shortest tooth on the apex. Leg completely light brown with numerous fine and long lateral tibial and femoral hairs (Fig. 10). Thorn-like hairs sparsely distributed on the proximal half of the femur and sometimes along tibial margins, but none on the tarsus.

Male nymph. Body length 10.52 ± 0.58 mm, abdominal length 4.75 ± 0.32 mm (Fig. 11A and 11B). Width of head/pronotal disc 2.12 ± 0.12/2.22 ± 0.16 mm (Fig. 11A and 11B). Ocelli 1.06 ± 0.28 mm diameters apart. Mandible 0.74 ± 0.03 mm (Fig. 9). Mesothoracic and metathoracic width 2.53 ± 0.14 mm and 2.43 ± 0.36 mm, respectively. Femur 1.67 ± 0.19 mm, tibia 1.59 ± 0.13 mm, tarsus 0.8 ± 0.07 mm (Fig. 10). Cercus 3.53 ± 0.35 mm.

Female nymph. Body length 14 ± 0.98 mm, abdominal length 6.54 ± 0.46 mm. Width of head against pronotal disc 2.73 ± 0.25/2.94 ± 0.17 mm, ocelli 1.5 ± 0.23 mm diameters apart, mandible 1.06 ± 0.07 mm. Mesothoracic and metathoracic width 3.31 ± 0.26 and 3.19 ± 0.28 mm, respectively. Femur 2.14 ± 0.35 mm, tibia 2.02 ± 0.29 mm, tarsus 1.02 ± 0.15 mm. Cercus 4.29 ± 0.48 mm.

Habitat. Adults of *P. constanspina* were collected from Layawan River (alt. 73–1,218 masl) of Mt. Malindang, northern Mindanao, Philippines. This fast flowing river has an average width of approximately 10 m, depth of 80 cm and is canopied with forest trees of mainly dipterocarps and palm species, as well as shrubs near the riverbank. Nymphs of *P. constanspina* are often sympatric with *Neoperla* species occurring under small to medium-sized cobbles, or can be found in leaf pack litter accumulated on rocks in fast currents.

Etymology. The species name refers to the constant array of fine, spine-like hairs of uniform length covering the apical tip of penial sac.

Association of sexes and life stages. The pairwise distances of *CO1* barcode sequences between males and presumptive female of *P. constanspina* revealed 100% similarity (no sequence divergence), suggesting that they belong to the same species. Between adults and presumptive nymphs, the sequence divergence is 0%, showing both life stages belong to the same species (see Table 2).

Diagnosis. The new species is a member of the *P. anomala* species complex and morphologically most similar to *P. bakeri*, known from the Philippines and Borneo. Similarities between *P. constanspina* and *P. bakeri* are in the structure of the penial capsule with a short, plump and unbranched penial tube. However, the everted sac of *P. constanspina* is distinguished by a blackened and rounded apical tip covered by small, spine-like hairs surrounding and covering the apex, where the jet-black hooks are positioned posteriorly forming a triangular shape, but with fewer hooks towards the base. *Phanoperla constanspina* has a droplet-shaped egg with long, slender collar, which is a typical in members of the *P. anomala* complex.

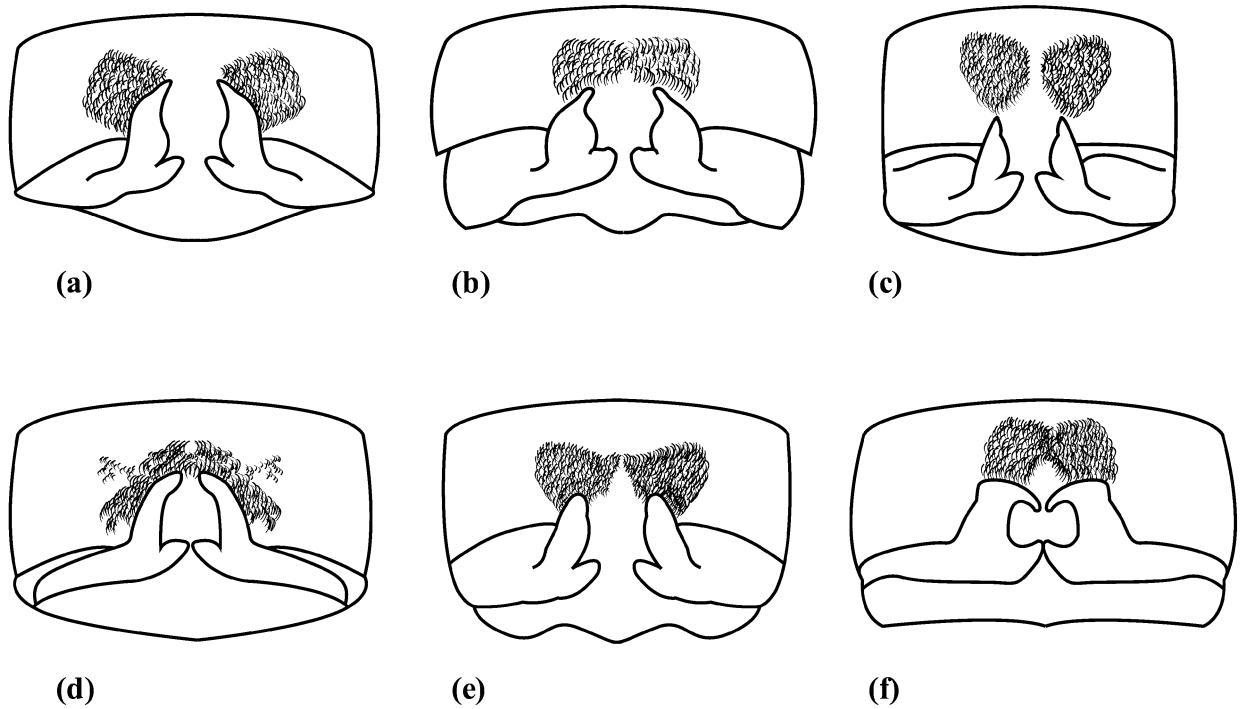


FIGURE 7. Phenotypic variation of six adult male *Phanoperla constanspina* **sp. nov**. based on the shape and structure of hemitergites (HT) and unbranched anterior process (AP), adorned with variable short setae on tergum 9. (A) (Haplotype: A-G23.m) Medium-sized AP, body and slightly bent on the pointed apex, setal patches isolated from each other. (B) (Haplotype: A-H7.m) AP small and pointed, setae located above the apical tip adjacent to each other. (C) (Haplotype: A-H16.m) Plump base and small AP (Fig. 5), with slightly pointed tip, paired setal patches adjacent and overlapping. (D) (Haplotype: A-H12.m) Medium and longer AP, bent on the inner side near the apex, setal patches fused, distributed along and near the process. (E) (Haplotype: A-E23.m) AP blunt on the tip in a medium-sized body, setal patches adjacent. (F) (Haplotype: A-H11.m) HT and AP broad and plump, resembling a hook-like structure at the tip, setal patches adjacent.

Discussion

The genus *Phanoperla* was distinguished from *Neoperla* by Zwick (1982) based on the direction of its Cu_2 from Cu_1 on the forewing which appears at an almost right-angle orientation (Fig. 2A). Also, the occiput of *Phanoperla* was described by Enderlein (1909) as possessing a well-marked median occipital line (Fig. 1A). These characters provide the reliable diagnostic characters for recognizing adult *Phanoperla*. The nymph of *Phanoperla* are separated from those of *Neoperla* by three diagnostic characters: 1) the presence of anterolateral submental lobes which are offset by horizontal basal suture; 2) the presence of only one pair of posterocoxal gills on the third coxa of the hindleg; and 3) the occurrence of setal fringes of small hairs on the posterior margin of the mesosternum (Zwick, 1982; Sivec *et al* 1988; Sivec & Yule, 2004).

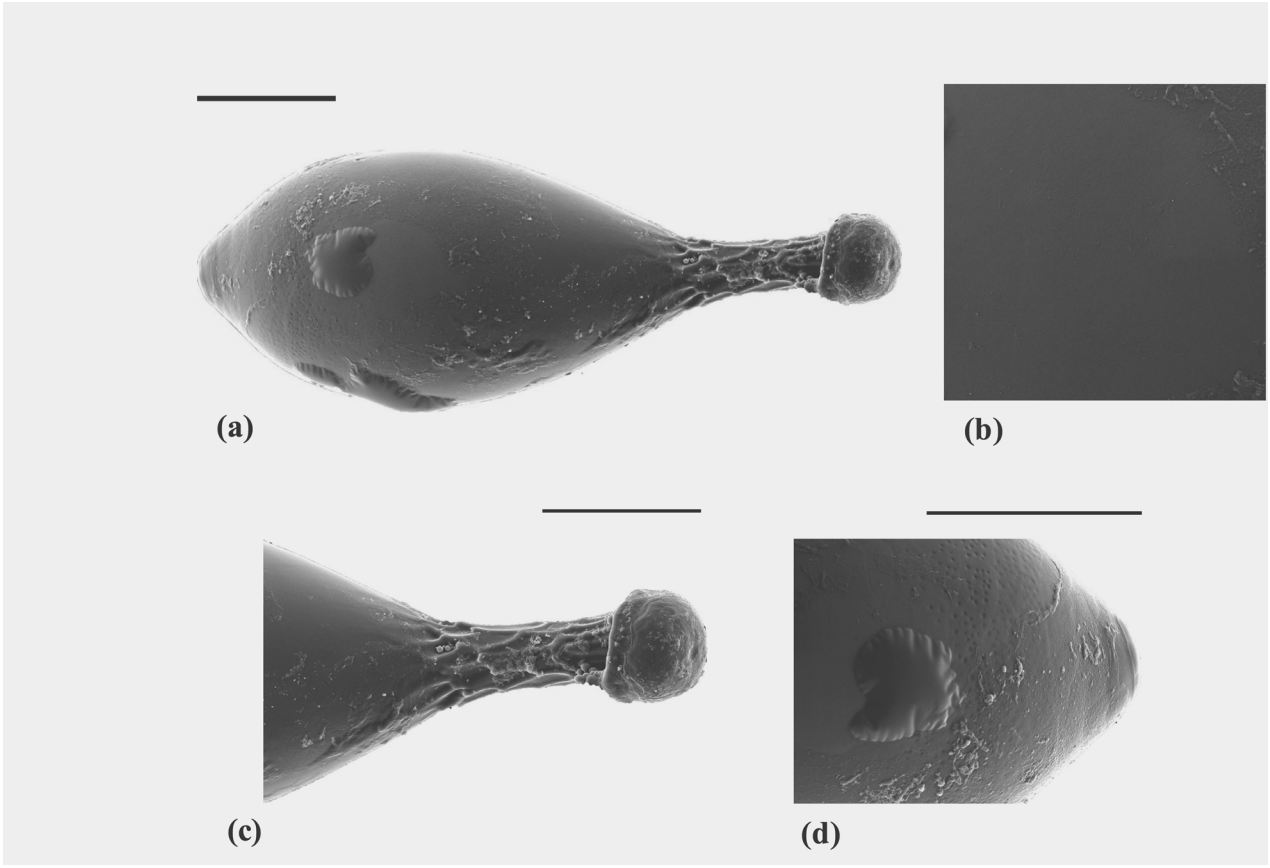
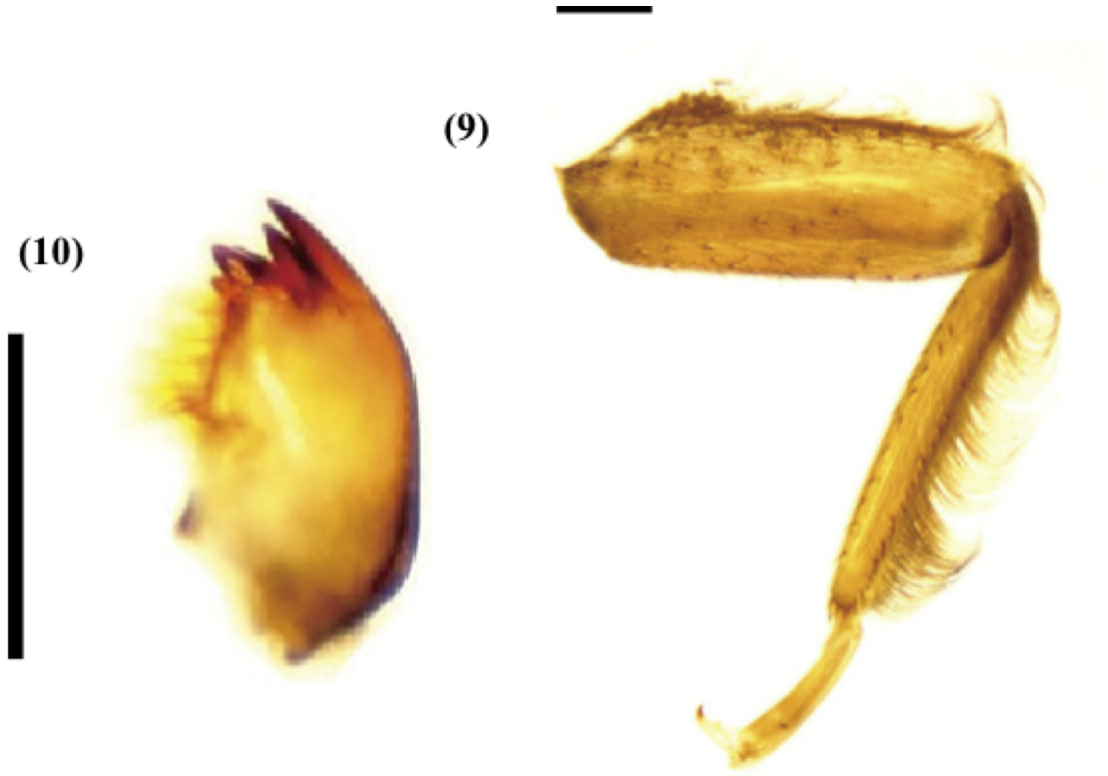


FIGURE 8. *Phanoperla constanspina* **sp. nov**. egg. (A) Entire egg. (B) Chorionic details. (C) Collar end (D) Anterior end. Scale = 100μm.



FIGURES 9–10. *Phanoperla constanspina* **sp. nov**. male nymph. (9) Right mandible. (10) Right foreleg. Scale = 0.5 mm.

We provide an identification key for *Phanoperla* species known from the Philippines based mainly on the detailed structure of the everted penial sac. This key was constructed from the description and illustrations of penial structures in Zwick (1982) and Sivec and Stark (2011). *Phanoperla claggi*, a species described from the Philippines is considered a *nomen nudum* and is excluded from the key. *Phanoperla omega* is relatively distinctive from the other Philippine *Phanoperla* because of its three-branched Rs (radial sector) of the forewing. *Phanoperla constanspina* is most similar to *P. batac* (Sivec and Stark, 2011), but it can be differentiated from *P. batac* by the absence of lobes in the penial sac and large black spines on the apex. Both *P. constanspina* and *P. batac* are closely related to *P. bakeri* and the three species belong to the *P. anomala* species complex, characterized by the droplet-shaped egg with a slender and elongated collar.

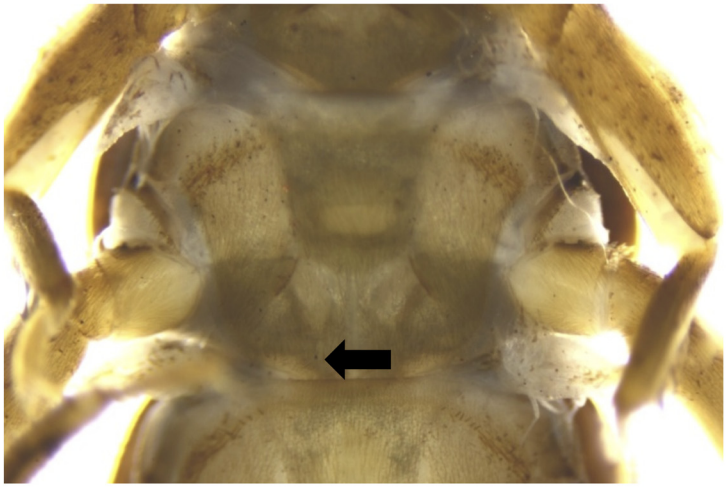
FIGURE 11. *Phanoperla constanspina* **sp. nov**. male nymphal habitus illustrating two general body pigmentation types. (A) (Haplotype: N-J25.m) Pale to lighter brown, sometimes with darker wingpad tips. (B) (Haplotype: N-G49.m). Dark to darker brown, sometimes with dark to black wingpads.

Key to known valid species *Phanoperla* of the Philippines. Characters of male everted penial sacs are adapted from the descriptions of Zwick (1982) and Sivec and Stark (2011).

1	Rs of forewing bifurcated into two branches, everted penial sac strongly widened towards apex	*P. omega*
1'	Rs of forewing with three branches	2
2	Penial sac consists of both basolateral rows and subapical ring of hooks in variable sizes, sometimes even fragmented	3
2'	Penial sac lacks basolateral rows, subapex armed with ringed black hooks or spines of variable sizes	5
3	Sac with wide tubular section and two small basolateral lobes; two short ventrolateral rows of very large hooks basally and apical ring	*P. flaveola*
3'	Sac without basolateral lobes or armature, but armed with prominent subapical ring and cylindrical structure on the apical tip	4
4	Penial sac with double ring of widely spaced, large black spines, mostly groups of three near the apex, few smaller spines near the sac base intermingled with larger black spines; cylinder armed over with black spines	*P. circumspina*
4'	Penial sac area around spine ring and lateral clusters of larger spines bearing numerous smaller spines; largest spines located in a cluster of eight along the ventral side of the subapical ring; cylinder much smaller than median basal section of sac, and covered with coarse to fine triangular spines	*P. magnaspina*
5	Everted internal sac curved to dorsal side; dorsal sclerite apically divided	*P. bakeri*
5'	Everted internal sac not curved, dorsal sclerite not apically divided	6
6	Penial tube bearing a thumb-shaped, apically directed spiny lobe and small spiny lobe near apex; complete ring of larger black spines on the apex with dense coating of small black spines beyond spine ring	*P. batac*
6'	Penial tube bare without lobes; black spines fine and small, covering the apex with constant length, surrounding the topmost of the apical surface; smaller spines posterior, becoming smallest towards the base	*P. constanspina*

(12A)

(12B)



(12C)

FIGURE 12. *Phanoperla constanspina* **sp. nov**. male nymph. (A) Suture of anterolateral submental lobes. (B) Mesosternum posterior margin pointing fringes of hairs. (C) One pair of posterocoxal gill of hindleg.

TABLE 2. Kimura-2-parameter (K2P) divergences between pair of CO1sequences of *Phanoperla constanspina* **sp. nov**. specimens.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	JN200437 *Agnetina annulipes*																			
2	JN200439 *Agnetina capitata*	13.3																		
3	JN200441 *Agnetina flavescens*	10.6	12.2																	
4	JN200654 *Neoperla clymene*	18.2	17.6	19.3																
5	JN200657 *Neoperla occipitalis*	20.9	19.2	19.2	16.3															
6	JN200658 *Neoperla stewarti*	20.9	18.8	19.8	19.3	16.0														
7	HQ152595 *Paragnetina immarginata*	22.0	22.1	19.9	24.9	22.1	20.3													
8	KT307709 *Neoperla layawanii*	24.9	22.4	23.3	24.3	24.7	26.0	24.6												
9	KT307712 *Neoperla longissimus*	22.5	22.6	23.0	23.5	23.8	22.7	22.5	20.5											
10	KT307715 *Neoperla malindang*	22.5	23.9	20.5	23.9	24.0	23.1	19.7	22.5	19.7										
11	**KT353027 *Phanoperla constanspina* sp. nov. adult male**	26.0	24.2	25.2	26.1	26.5	26.1	21.6	28.3	26.7	22.3									
12	**KT353028 *Phanoperla constanspina* sp. nov. adult male**	26.0	24.2	25.2	26.1	26.5	26.1	21.6	28.3	26.7	22.3	**0.0**								
13	**KT353025 *Phanoperla constanspina* sp. nov. adult male**	26.0	24.2	25.2	26.1	26.5	26.1	21.6	28.3	26.7	22.3	**0.0**	**0.0**							
14	**KT353023 *Phanoperla constanspina* sp. nov. adult male**	26.0	24.2	25.2	26.1	26.5	26.1	21.6	28.3	26.7	22.3	**0.0**	**0.0**	**0.0**						
15	**KT353026 *Phanoperla constanspina* sp. nov. adult male**	26.0	24.2	25.2	26.1	26.5	26.1	21.6	28.3	26.7	22.3	**0.0**	**0.0**	**0.0**	**0.0**					
16	**KT353024 *Phanoperla constanspina* sp. nov. adult male**	26.0	24.2	25.2	26.1	26.5	26.1	21.6	28.3	26.7	22.3	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**				
17	**KT353022 *Phanoperla constanspina* sp. nov. adult female**	26.0	24.2	25.2	26.1	26.5	26.1	21.6	28.3	26.7	22.3	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**			
18	**KT353021 *Phanoperla constanspina* sp. nov. nymph male**	26.0	24.2	25.2	26.1	26.5	26.1	21.6	28.3	26.7	22.3	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**		
19	**KT353019 *Phanoperla constanspina* sp. nov. nymph male**	26.0	24.2	25.2	26.1	26.5	26.1	21.6	28.3	26.7	22.3	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	

TABLE 3. Checklist of the *Phanoperla* (Plecoptera: Perlidae) endemic to the Oriental Region.

No.	Taxa	Species-group complex established by Zwick 1982	Distribution	Source
1	*P. amorpha* Zwick 1982	*incertae sedis***	India	Zwick 1982
2	*P. anomala* Banks 1939	*anomala*	Borneo	Zwick 1982
3	*P. astrospinata* 2009	*flaveola*	Brunei	Stark & Sheldon 2009
4	***P. bakeri* Banks 1924**	***anomala***	**Philippines, Borneo**	**Zwick 1982**
5	***P. batac* Sivec & Stark 2011**	***anomala***	**Philippines**	**Sivec & Stark 2011**
6	*P. belalong* Stark & Sheldon 2009	*	Brunei	Stark & Sheldon 2009
7	*P. ceylonica* Kawai 1975	*testacea*	Sri Lanka	Zwick 1982
8	***P. circumspina* Sivec & Stark 2011**	***flaveola***	**Philippines**	**Sivec & Stark 2011**
9	***P. claggi* Banks 1938**	***nomen nudum*****	**Philippines**	**Zwick 1982**
10	***P. constanspina* sp. nov. dela Cruz *et al* 2015**	***anomala***	**Philippines**	**This study**
11	*P. cornuta* Zwick 1982	*maindroni*	India	Zwick 1982
12	*P. doisuthep* Sivec & Stark 2010	*	Thailand	Sivec & Stark 2010
13	*P. flabellare* Stark & Sivec 2007	*	Kalimantan	Stark & Sivec 2007
14	***P. flaveola* Klapalek 1910**	***flaveola***	**Philippines, Borneo, Java, Kalimantan**	**Zwick 1982, Stark & Sivec 2007**
15	*P. fuscipennis* Navas & Zwick 1983	*	Vietnam	Zwick 1983, Sivec & Stark 2010
16	*P. guttata* Zwick 1982	*nervosa*	Sumatra	Zwick 1982, Zwick & Sivec 1985
17	*P. himalayana* Zwick 1977	*maindroni*	India, Himalayas	Zwick 1982
18	*P. hubleyi* Sivec & Stark 2010	*	Vietnam	Sivec & Stark 2010
19	*P. huang* Sivec & Stark 2010	*pallipennis*	Thailand	Sivec & Stark 2010
20	*P. imitatrix* Zwick 1986	*	Vietnam	Zwick 1986, Sivec & Stark 2010
21	*P. incompleta* Zwick 1986	*nervosa*	Borneo	Zwick 1986, Sivec & Stark 2010
22	*P. lao* Stark 1983	*	Thailand	Stark 1983, Sivec & Stark 2010
23	*P. limosa* Hagen 1858	*testacea*	Sri Lanka	Zwick 1982
24	*P. lisu* Stark 1983	*pallipennis*	Thailand	Stark 1983, Sivec & Stark 2010
25	*P. lobata* Sivec & Stark 2010	*	Vietnam	Sivec & Stark 2010
26	*P. maculata* Zwick 1982	*nervosa*	Borneo	Zwick 1982
27	***P. magnaspina* Sivec & Stark 2011**	***flaveola***	**Philippines**	**Sivec & Stark 2011**
28	*P. maindroni* Navas 1926	*maindroni*	India	Zwick 1982
29	*P. malayana* Zwick 1982	*pallipennis*	Thailand, Malaysia	Zwick 1982, Sivec & Stark 2010

……continued on the next page

TABLE 3. (Continued)

No.	Taxa	Species-group complex established by Zwick 1982	Distribution	Source
30	*P. minutissima* Enderlein 1909	*testacea*	Sumatra	Zwick 1982, Zwick & Sivec 1985
31	*P. namcattien* Bae et al 2009	*	Vietnam	Bae et al 2009
32	*P. nana* Zwick 1982	*nana*	Sri Lanka	Zwick 1982
33	*P. nervosa* Banks 1939	*nervosa*	Borneo	Zwick 1982
34	*P. nuwara* Kawai 1975	*testacea*	Sri Lanka	Zwick 1982
35	*P. occipitalis* Sivec & Stark 2010	*	Thailand	Sivec & Stark 2010
36	***P. omega* Zwick 1982**	***nervosa***	**Philippines**	**Zwick 1982**
37	*P. pallipennis* Banks 1938	*pallipennis*	Hainan Island PRC	Zwick 1982
38	*P. parva* Zwick 1982	*nana*	India	Zwick 1982
39	*P. peniculus* Kawai 1969	*flaveola*	India	Zwick 1982
40	*P. pumilio* Klapalek 1921	*nervosa*	Borneo, Kalimantan, Brunei	Zwick 1982, Stark & Sivec 2007, Sivec & Stark 2010
41	*P. schmidi* Zwick 1982	*incertae sedis***	India	Zwick 1982
42	*P. sertispina* Jewett 1975	*anomala*	India, Thailand	Zwick 1982, Stark 1983
43	*P. simplex* Zwick 1982	*testacea*	Thailand, Malaysia, Sumatra	Zwick 1982, Zwick & Sivec 1985, Sivec & Stark 2010
44	*P. srilanka* Zwick 1982	*testacea*	Sri Lanka	Zwick 1982
45	*P. sumatrae* Zwick 1982	*flaveola*	Sumatra	Zwick 1982, Zwick & Sivec 1985
46	*P. testacea* Zwick 1982	*testacea*	Sri Lanka	Zwick 1982
47	*P. tuberosa* Stark & Sivec 2007	*	Kalimantan	Stark & Sivec 2007
48	*P. uchidai* Sivec & Stark 2010	*	Thailand	Sivec & Stark 2010
49	*P. vietnamensis* Zwick 1986	*flaveola*	Vietnam	Zwick 1986, Sivec & Stark 2010
50	*P. wedda* Zwick 1982	*testacea*	Sri Lanka	Zwick 1982
51	*P. weing* Sivec & Stark 2010	*	Thailand	Sivec & Stark 2010

Note: Species-groups as established by Zwick (1982) have not been assigned to some species and are indicated by *, whereas *incertae sedis*** indicates unclassified to the given group complexes. Distribution is based on type locations of the species.

Male genitalia provide the primary diagnostic characters for species separation in *Phanoperla.* Zwick (1982) indicated several *Phanoperla* species can be sympatric from the same locale, but the association of sexes of the same species is either difficult or problematic, as in the case of *P. parva* Zwick 1982 in India, and even impossible, e.g. *Phanoperla* members from *testacea*-group in Sri Lanka. In this study, a few taxonomic problems were encountered from mismatching of adult males with females; therefore a number of females could not be positively associated with males (Zwick, 1982). Here, we evaluated the utility of DNA barcoding for identifying morphologically ambiguous nymphs and females of *P. constanspina*. Six haplotypes of intraspecific male adults with identical penial structures, but with different morphological characters of tergal tips, particularly the shape and structure of hemitergite and anterior process (Fig. 7) were analyzed. Two haplotypes of intraspecific male nymphs of similar developmental stage possessing different overall body pigmentation (Fig. 11) and exhibit variable hemitergal structures within species, but were shown to have 0% divergence of DNA barcode sequences. Similarly, the two nymphal traits of body pigmentation in *P. constanspina* are intraspecific variation. Our findings indicate that morphological variation of taxonomically important characters can pose challenges during species identification. The ability to use DNA barcoding sequence data for associating and verifying specimens of different life stages and sexes is useful for *P. constanspina*. Similar success may be expected in associating other *Phanoperla* species occurring in the Philippines.

Table 3 includes a checklist of all 51 currently recognized species of *Phanoperla* restricted to the Oriental Region. Although biogeographically, these taxa are endemic to the Oriental Region, most species are known from a single island or from restricted areas of the mainland. Zwick (1982) reported that a few of these species perhaps are expanding their range. Adults of *Phanoperla* are weak flyers, but widespread species are known. For example, species such as *P. sertispina* Jewett 1975 occur both in southern India and Thailand. The Philippine species, *P. bakeri* and *P. flaveola*, both occurring also in Borneo, are hypothesized to have colonized their insular ranges during Pleistocene glaciations when the present islands were connected by land bridges (Zwick 1982). Within their archipelagic range, *P. bakeri*, *P. flaveola*, and *P. omega* are reported from Luzon of northern Philippines, Mindanao Island of the south, and Palawan Islands of the Southwest.

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