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A new record of *Neoperla obliqua* Banks, 1930 (Plecoptera: Perlidae) from Mt. Malindang, Mindanao, Philippines and association of life stages using DNA barcodes

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Abstract

The nymph of the perlid *Neoperla obliqua* Banks, 1913 from Mt. Malindang, Mindanao Island, the Philippines is described and associated with the male and female adults using DNA barcodes. Using pairwise *COI*, the nymph was associated with $99.9 \pm 0.14\%$ interspecific similarity, while comparison between sexes generated a 0.2% intraspecific divergence between males and females of putative conspecifics. Additionally, the mature ova from female adults are described using scanning electron microscopy (SEM). The distinctive chorionic surface is rugose with longitudinal ridge-like pattern with a bare and flat collar.

Key words: DNA barcode, *Neoperla*, nymph, ova, Philippines, Plecoptera

Introduction

The Philippine Archipelago is comprised of more than 7,000 oceanic islands and is one of the world's most species rich areas (Brown & Diesmos, 2009) due to its geographic isolation, ancient geological histories, ecological gradients, and the repeated formations of Pleistocene Aggregate Island Complexes (PAICs) resulting from past climatic oscillation of sea levels (Brown *et al.*, 2013). The Archipelago has a distinct species assemblage of the Perlidae genus *Neoperla*. These species exhibit substantial geographical dissimilarity both between and within individual islands (Sivec, 1984). The species of *Neoperla* are primarily distributed in the Philippines in Mindanao and northern Luzon, with one species reported from the Bicol of southern Luzon. Several species also occur in central Philippines, particularly on Samar, Leyte, Cebu, Negros, and Palawan. Other studies of Oriental *Neoperla*, particularly of Java, Sumatra, and Borneo are available (Zwick 1983, 1986). Sivec (1984) and Sivec & Stark (2011) presented reviews of the Philippine *Neoperla*, studying types and other available material, characterizing at least 23 species using aedeagal, vaginal sclerites, and ova chorion structures (Table 4).

The studies by Sivec (1984) and Sivec and Stark (2011) have provided a firm basis for the further studies of Philippine *Neoperla* that identified species through the shape of the aedeagus and the size, and the distribution of spines that occur on penial sac. *Neoperla pallicornis* Banks, 1937 from Mt. Makiling and Mt. Montalban, as well as from Samar and Leyte, Philippines is known only from females. A preliminary arrangement of *Neoperla* species into groups and subgroups of species complexes by Zwick (1983) was followed by Sivec (1984). The oculata species complex currently contains 11 species including *N. obliqua* Banks, 1913. *Neoperla obliqua* is among the most common perlid species in the Philippines. The previous association of both sexes of this species was based on sympatric distributions, size and general habitus (Sivec, 1984). Reported Philippine records of *N. obliqua* were from different areas of the Archipelago (Sivec, 1984). Within Mindanao, the species was reported from the mountains of Bukidnon, Surigao, Misamis, Agusan, Davao, and Zamboanga, particularly based on female

specimens (Sivec, 1984). However, several female specimens of this species were available from Mt. Makiling, Laguna of Luzon.

The association of all life stages of Philippine *Neoperla* is incomplete. Here, we associate for the first time the nymph of *N. obliqua* using DNA barcodes from Mt. Malindang, Mindanao, Philippines, a new locality record for this species. Additionally, we illustrate the fine details of ova of this species using scanning electron microscopy.

Material and methods

Adult stoneflies were collected using light traps and the nymph was handpicked from rocks and cobble of a shallow reach of the Layawan River, Mt. Malindang Range Natural Park, Mindanao Island, Philippines. The specimens were immediately placed in 95% ethanol for subsequent examination and DNA analysis. Specimens were examined using a stereo microscope (Olympus SZ51 & SZ61, Tokyo, Japan) with an attached digital camera (Canon EOS700, Tokyo, Japan) at 0.67-4.5x magnifications for imaging. Morphological structures were illustrated using Adobe Illustrator CS6 (v.16.0.0, Adobe Systems Inc., San Jose, CA, USA). Measurements of the morphological structures were made using ImageJ (v.1.48, National Institutes of Health, Bethesda, Maryland, USA). The ova was studied using a Hitachi, SU1510 (Tokyo, Japan) scanning electron microscope (SEM). Morphological terminology follows Sivec (1984) and Sivec & Stark (2011).

DNA extraction, PCR (Polymerase Chain Reaction) amplification, and sequencing follow Lin & Wood (2002). The animal DNA barcode, *COI* (*cytochrome c oxidase subunit I*) were amplified by universal primers, LCO1490 and HCO2198 (Folmer *et al.*, 1994). Each PCR reaction contained 5 µL of TAE buffer, 35 µL of ddH₂O, 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 using the BigDye® terminator 3.1 sequencing kit on an ABI 3730XL DNA Analyzer (Applied Biosystems). The DNA sequences of between 583 and 618 base pairs were obtained (GenBank accession numbers, Table 1), and then assembled manually using SeqMan Pro of DNASTAR (v.7.1.0, Lasergene, Madison, WI, USA) and aligned using Clustal W in MegAlign of DNASTAR. The Genbank numbers were generated from the Barcode of Life Data System (BOLD v.4) while the nucleotide sequence of related species were deposited from National Center for Biotechnology Information (NCBI). Intraspecific and interspecific sequence similarities of available perlid stoneflies were calculated using pairwise distance of Kimura-2-parameter (K2P) model of nucleotide substitution.

TABLE 1. GenBank accession numbers of the collected Philippine *Neoperla* species from Mt. Malindang, Philippines.

Taxon	Haplotype Code	Sex	Life Stage	Accession Number
<i>Neoperla obliqua</i>	A-I1.m	♂	Adult	KT307712
<i>Neoperla obliqua</i>	A-G10.f	♀	Adult	KT307713
<i>Neoperla obliqua</i>	N-A1.f	♀	Nymph	KT307714

Results

Neoperla obliqua, Banks 1913

Material examined. Philippines, Mindanao, Mt. Malindang, Layawan River. 1 ♂, 127masl, N 08°26.314' E 123°42.492', 5 October 2013; 3 ♀, 185masl, N 08°25.418' E 123°41.806', 3 October 2013. 185masl, N 08°25.418' E 123°41.806', 3 October 2013; 1 ♀ nymph, 1,218masl, N 08°18.495' E 123°37.980'. All material is deposited in the Natural Science Museum, MSU-IIT, Iligan City, Philippines.

Ova. Shape oval, highly striated, (Fig. 1A). Collar present but flat. Length ca. 318.16 ± 10.37 µm (n=3), equatorial width ca. 222.63 ± 4.97 µm. Collar bare, with length ca. 16.2 ± 8.5 µm and width ca. 85.83 ± 9.7 µm, with uneven surface, resembling a lid (Fig. 1C). Chorionic surface rugose and somewhat ornate. Gyral structure

irregular but usually follows a longitudinal ridge-like pattern which arises from the collar end, creating a sulci or irregular grooves in between studded with pores of almost similar sizes (Fig. 1D). Micropyles at distal end of the collar and present in all ova examined (Fig. 1B).

Nymph. General color golden ochre to pale orange, while darker, usually dark brown to black margins on frons and labrum of head, the pronotal disc, tip of wingpads, and abdominal terga (Fig. 2A). Pronotal disc oval-shaped. Wingpads not elongated. Terga faintly striped. Antennae and cerci setaceous. Mandible ochre, ovate-shaped but broad on its base possessing a five comb-like, blunted teeth of irregular sizes (Fig. 2B). Few fine setal hairs on the inner edges of mandible. Legs golden-brown without acute setal surface hairs (Fig. 2C).

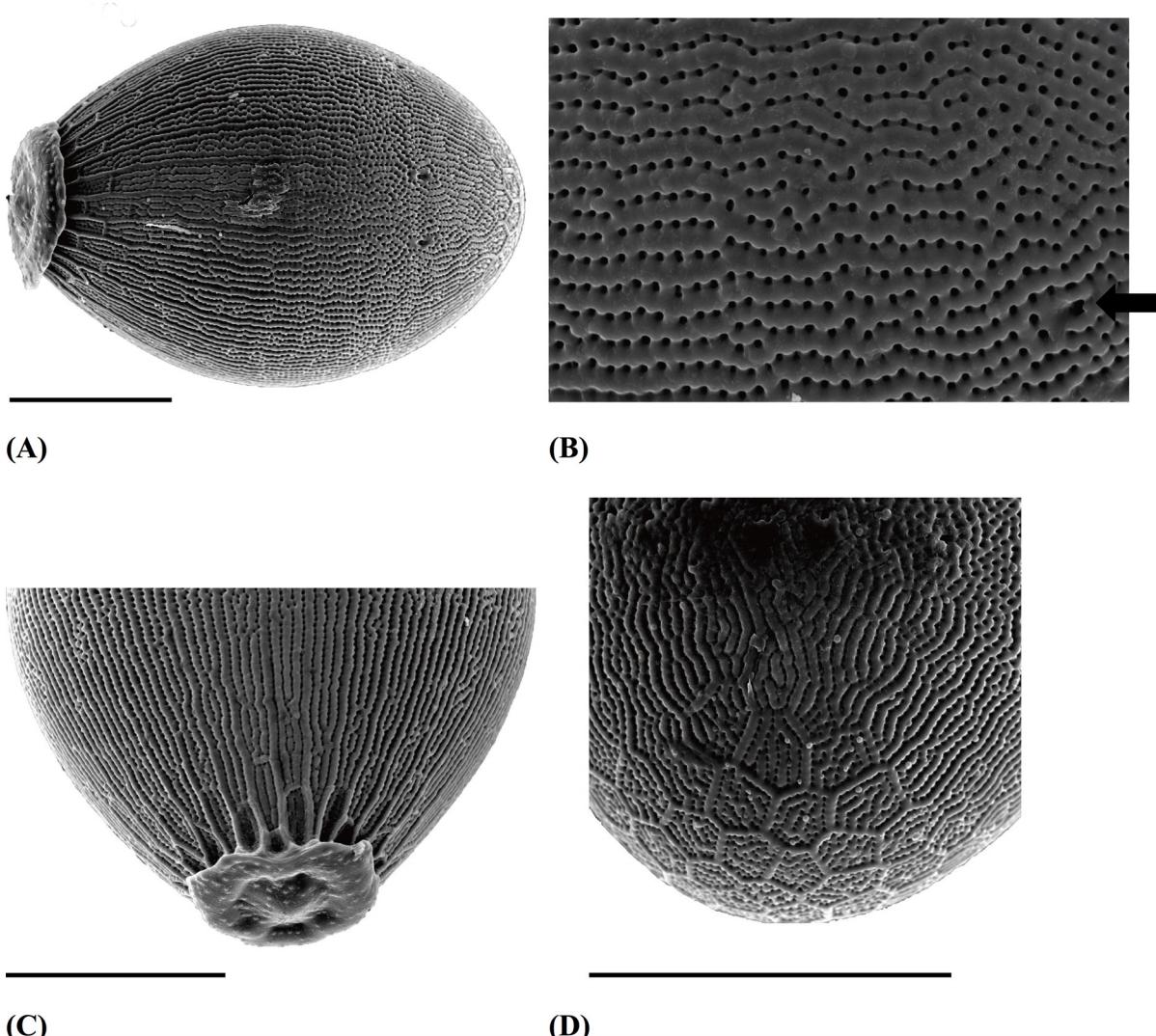


FIGURE 1. *Neoperla obliqua* ova. (1A) Entire ova. (1B) Chorionic details (arrow refers to micropyle). (1C) Collar end. (1D) Anterior end. Scale = 100 μ m.

Female nymph. Body length of 17.44 mm (n=1), abdominal length 7.02mm. Width of head is 4.01mm, pronotal disc is 3.91mm. Ocelli 0.8mm diameters apart. Mandible 1.43 mm. Mesothoracic and metathoracic width 4.36 mm and 4.07 mm, respectively. Antenna 11.05 mm. Femur 4.13 mm, tibia 3.94 mm, tarsus 1.22 mm. Cercus 9.78 mm.

Habitat. Adults of *N. obliqua* were sympatric with another unidentified perlid stonefly species. The single nymph of *N. obliqua* was found under large rocks in the Layawan River, in a section with moderate current.

Life stage and sex association. Pairwise *COI* sequence comparisons revealed 0.2% intraspecific divergence between males and females of putative conspecifics. The sequence similarity between adult and nymphal stages was $99.9 \pm 0.14\%$ (Table 2). Comparisons between divergences of interspecific *COI* sequences of all available perlid stoneflies based on Kimura-2-parameter (K2P) model were also shown (Table 3).

TABLE 2. Divergence of intraspecific *COI* sequences of *Neoperla obliqua* based on Kimura-2-parameter (K2P) model.

	1	2	3
1	KT307712 <i>N. obliqua</i> male adult		
2	KT307713 <i>N. obliqua</i> female adult	0.2	
3	KT307714 <i>N. obliqua</i> female nymph	0	0.2

TABLE 3. Divergence of interspecific *COI* sequences of available Perlidae stoneflies based on Kimura-2-parameter (K2P) model.

	1	2	3	4	5	6	7	8
1	JN200676 <i>Paragnetina immarginata</i>							
2	JN200438 <i>Agnetina annulipes</i>	20.6						
3	JN200439 <i>Agnetina capitata</i>	22.4	13.6					
4	JN200441 <i>Agnetina flavescens</i>	19.8	10.9	12.2				
5	JN200654 <i>Neoperla clymene</i>	25.9	18.0	17.6	19.3			
6	JN200657 <i>Neoperla occipitalis</i>	21.8	19.3	19.2	19.2	16.3		
7	JN200658 <i>Neoperla stewarti</i>	19.8	20.5	18.8	19.8	19.3	16.0	
8	KT307712 <i>Neoperla obliqua</i>	22.1	21.5	22.6	23.0	23.5	23.8	22.7

TABLE 4. Checklist of the Philippine *Neoperla* species.

Taxa	species-complex	Materials available		Source
		Adult	Nymph	
<i>N. agtouganon</i> Sivec & Stark 2011	oculata	♂ ♀	unknown	Sivec & Stark 2011
<i>N. agusani</i> Sivec 1984	incertae sedis	♂	unknown	Sivec 1984
<i>N. andreas</i> Sivec & Stark 2011	recta	♂	unknown	Sivec & Stark 2011
<i>N. atropennis</i> Banks 1924	oculata	♂ ♀	unknown	Jewett 1958; Sivec 1984
<i>N. connectens</i> Zwick 1986	oculata	♂	unknown	Sivec 1984; Zwick 1986
<i>N. dentata</i> Sivec 1984	variegata	♂ ♀	unknown	Sivec 1984
<i>N. flinti</i> Sivec 1984	oculata	♂ ♀	unknown	Sivec 1984
<i>N. hermosa</i> Banks 1924	oculata	♂ ♀	unknown	Sivec 1984
<i>N. jewetti</i> Sivec 1984	oculata	♂	unknown	Jewett 1958; Sivec 1984
<i>N. nigra</i> Sivec 1984	oculata	♂	unknown	Sivec 1984
<i>N. nishidai</i> Sivec 1984	recta	♂ ♀	unknown	Sivec 1984
<i>N. obliqua</i> Banks 1913	oculata	♂ ♀	known	Jewett 1958; Sivec 1984
<i>N. oculata</i> Banks 1924	oculata	♂	unknown	Jewett 1958; Sivec 1984
<i>N. palawan</i> Sivec & Stark 2011	oculata	♂	unknown	Sivec & Stark 2011
<i>N. pallescens</i> Banks 1924	oculata	♂ ♀	unknown	Sivec 1984
<i>N. pallicornis</i> Banks 1937	oculata	♀	unknown	Sivec 1984
<i>N. philippina</i> 1984	oculata	♂	unknown	Sivec 1984
<i>N. pseudorecta</i> Sivec 1984	recta	♂	unknown	Sivec 1984
<i>N. recta</i> Banks 1913	recta	♂ ♀	unknown	Jewett 1958; Sivec 1984
<i>N. sabang</i> Sivec & Stark 2011	oculata	♂ ♀	unknown	Sivec & Stark 2011
<i>N. salakot</i> Sivec & Stark 2011	oculata	♂ ♀	unknown	Sivec & Stark 2011
<i>N. wagneri</i> Sivec 1984	oculata	♂	unknown	Sivec 1984
<i>N. zwicki</i> Sivec 1984	recta	♂	unknown	Sivec 1984

Discussion

The nymph of *N. obliqua* is associated positively with our adults and described for the first time. The ova as described by Sivec (1984) were identical to the SEM images of the new record of *N. obliqua* from Layawan River. Ova were taken from extruded egg masses at the tip of the abdomen of the collected females. Our new SEM images supplement the description by Sivec (1984).



FIGURE 2. *Neoperla obliqua* ♀ nymph. (2A) Nymphal habitus. (2B) Right mandible. (2C) Right foreleg. Scale = 1.00 mm, except for mandible which is 0.5 mm.

Since the pioneer works of Banks in the 1930's, the *Neoperla* of the Philippine Archipelago has been studied by Sivec (1984) and Sivec and Stark (2011). A total of 23 *Neoperla* species are currently known from the region (Table 5). In the Philippines, The *N. oculata* species complex is the most species-rich group, consisting of more than 70% (17 species) of the total number of *Neoperla* species, followed by the *N. recta* species complex (5 species), *N. variegata* species complex (1 species) and one *incertae sedis* species. We associated the nymph of *N. obliqua* which were previously unknown. Furthermore, the availability of integrated taxonomic resolution in both morphological and molecular characters through DNA barcodes may also offer a better understanding to the systematics and evolution of this Philippine perlid species.

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