



## Larvae and females of two *Stenopsyche* species in Taiwan with redescription of the male of *S. formosana* (Insecta: Trichoptera)

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### Abstract

Larvae and females of two *Stenopsyche* species, *S. formosana* Kobayashi and *S. drakon* Weaver, are described for the first time. They were associated with the adult male through mitochondrial DNA sequence comparisons. The male of *S. formosana*, which has been recorded as *Stenopsyche banksi* Mosely from Taiwan, is redescribed. The males of *S. formosana* and *S. banksi* can be distinguished from each other by the shape of segments IX and X, and endothecal spines. In larvae, *S. formosana* and *S. drakon* are distinguishable from each other and from other known species by the combination of different dark marking patterns and shapes of no. 18 setae of the head, and by the shapes of forecoxae. The larval morphology of *S. formosana* and *S. drakon* suggests that the phylogenetic relationships based mainly on male genitalia in the genus *Stenopsyche* should be reconsidered.

**Key words:** Stenopsychidae, *Stenopsyche formosana*, *Stenopsyche drakon*, mitochondrial DNA, association

### Introduction

The genus *Stenopsyche* McLachlan is a genus composed of more than 90 species distributed in the East Palearctic, Oriental and Afrotropical regions (Morse 2016). Larvae of this genus are a distinct element of the fauna of Asian rivers and streams (Dudgeon 1999), however, the larvae of most species are not yet known (Xu *et al.* 2015). Knowledge about females of this genus is also poor (Xu *et al.* 2015). In Taiwan, Yang *et al.* (2005) listed seven *Stenopsyche* species: *S. banksi* Mosely 1942, *S. drakon* Weaver 1987, *S. formosana* Kobayashi 1987, *S. pallidipennis* Martynov 1926, *S. pjasetzkyi* Martynov 1914, *S. sauteri* Ulmer 1907, and *S. taiwanensis* Weaver 1987. Furthermore, Malicky (2014) recorded three additional species: *S. arvadit* Malicky 2008, *S. remihel* Malicky 2014 and *S. schmidi* Weaver 1987. However, larvae and females of these species have not yet been described.

In 2015, adults and larvae of various aquatic insects were collected from rivers in Nantou County in Taiwan (Nishimura *et al.* 2015). In the collection, we found males of two *Stenopsyche* species, *S. formosana* and *S. drakon*, and also two discrete morphotypes of larvae and females, respectively. Each morphotype of these larvae and females was associated through DNA sequence comparisons with the males. In this paper, we describe larvae and females of these two *Stenopsyche* species, and redescribe the male of *S. formosana* which has been recorded as a continental species *S. banksi* (Mosely 1942; Biodiversity Research Center, Academia Sinica 2015).

### Materials and methods

**Specimens.** DNA sequences of eight specimens of *Stenopsyche* caddisflies from two localities in Taiwan were

analyzed for the DNA sequences at the mitochondrial COI region (636-bp.). We analyzed males of *Stenopsyche formosana* (specimen No. 1: Table 1) and *Stenopsyche drakon* (specimen Nos. 2–3: Table 1) identified by their morphological characteristics as the reference specimens. We also analyzed two females (specimen Nos. 4–5: Table 1) and three larvae (specimen Nos. 6–8: Table 1) of *Stenopsyche* caddisflies which have not been associated with their adult males. Some additional specimens were used for morphological study.

**TABLE 1.** Specimen information for *Stenopsyche* spp. used for molecular phylogenetic analyses.

Sample No.	Locality (Ren'ai Xiang, Nantou County)	Latitude	Longitude	Altitude (m)	Stage	GenBank Accession No.
1	Beigang River, Huisun Forest Area	24°4.992'	121°0.651'	490	Male	LC109674
2	Beigang River, Huisun Forest Area	24°4.992'	121°0.651'	490	Male	LC109678
3	Meiyuan River	24°5.09'	120°57.22'	504	Male	LC109679
4	Meiyuan River	24°5.09'	120°57.22'	504	Female	LC109675
5	Beigang River, Huisun Forest Area	24°4.992'	121°0.651'	490	Female	LC109680
6	Meiyuan River	24°5.09'	120°57.22'	504	Larva	LC109676
7	Meiyuan River	24°5.09'	120°57.22'	504	Larva	LC109677
8	Beigang River, Huisun Forest Area	24°4.992'	121°0.651'	490	Larva	LC109681

**Morphological study.** Male and female genitalia were figured after being cleared in a 10% solution of KOH. Photographs of larval characters were taken using Nikon Coolpix P340 through Olympus SZX 12 stereomicroscope, and combined by the image stacking software Combine ZP. Larval characters described are based on final instar larvae. Morphological terms mostly follow Xu *et al.* (2015). Setal nomenclature is based on that of Williams & Wiggins (1981) and Nozaki & Shimura (2015). Depositories of specimens are abbreviated as follows: Natural History Museum and Institute, Chiba, Japan (CBM); Shinshu University, Matsumoto, Japan (SU) and T. Nozaki, Ninomiya, Kanagawa, Japan (TN).

**DNA extraction, amplification, sequencing and alignment.** Total genomic DNA was extracted from ethanol preserved tissues of specimens and purified using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden), according to the manufacturers' instructions. Total genomic DNA was used to amplify DNA fragments [the mitochondrial DNA (mtDNA) cytochrome c oxidase subunit I (COI) by polymerase chain reaction (PCR) with sets of primers [LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3', and HCO2198: 5'-TAAACTTCAGGGTGACCAAAA AATCA-3' (Folmer *et al.* 1994)]. The PCR protocol was: 94°C for 1 min; 35× (94°C for 1 min, 45°C for 2 min, 72°C for 1 min); 72°C for 7 min. PCR products were purified using the illustra ExoProStar (GE Healthcare, Buckinghamshire). Purified DNA fragments were sequenced directly by an automated method using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, California) on an automated DNA Sequencer (ABI 3130 or 3130xl DNA Analyzer; Perkin Elmer/Applied Biosystems, California). All of the sequence data have been submitted to the DNA data bank of Japan (DDBJ database), and the accession numbers are given in Table 1. As outgroups, we have utilized the sequence data of the two philopotamid caddisflies: *Chimarra marginata* (GenBank accession No. FM998346) and *Chimarra social* (GU114726). In addition, we have also used the sequence data of East Asian *Stenopsyche* species listed below, which have been registered in GenBank: *S. marmorata*, *S. schmidi* and *S. sauteri* (GenBank accession No. LC109682–109695).

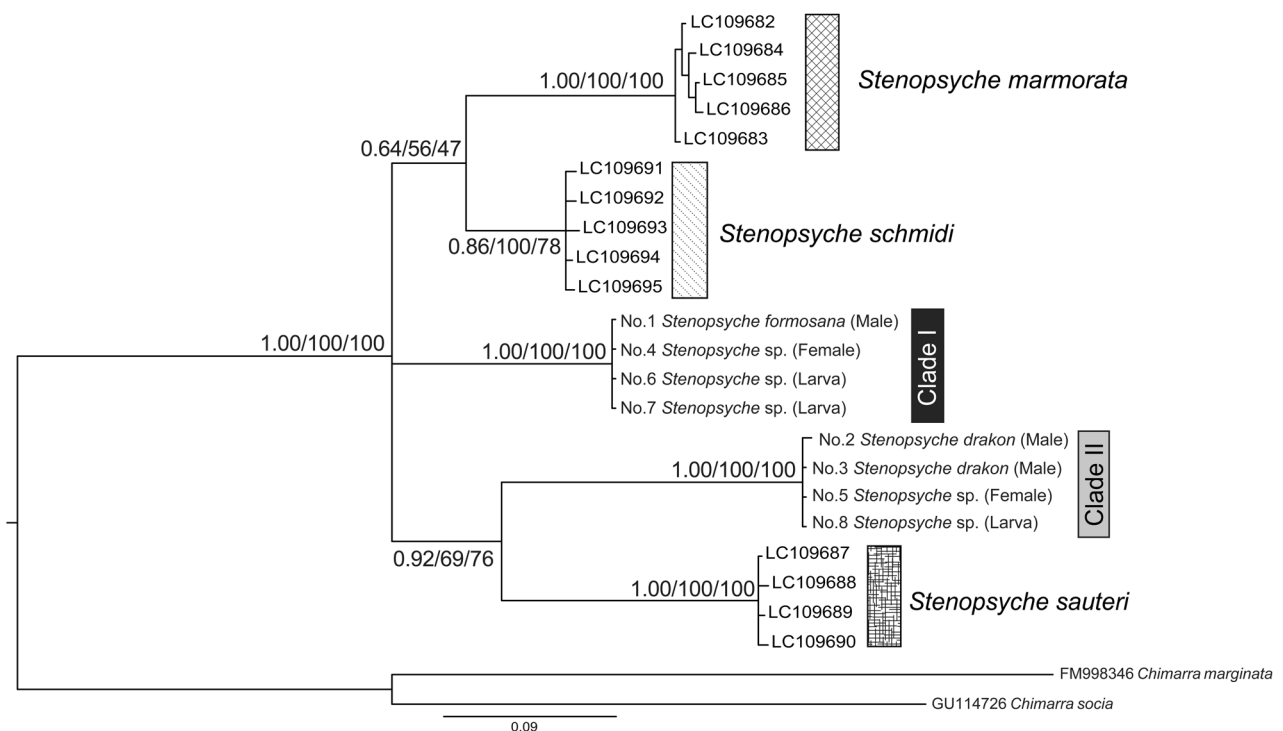
Sequence alignment and editing were performed for each gene separately using MEGA version 5.02 (Tamura *et al.* 2011) and CLC Workbench software (CLC bio, Aarhus). All sequence data were aligned automatically with Clustal W (Thompson *et al.* 1994) and MEGA version 5.02. The alignments were determined for unique haplotypes using the software DnaSP v4.0 (Rozas *et al.* 2003) prior to subsequent analysis.

**Phylogenetic analysis.** Phylogenetic analyses were performed by neighbor joining (NJ: Saitou & Nei 1987) and maximum likelihood (ML: Felsenstein 1981) using MEGA version 5.02 with 1,000 bootstrap replications. Prior to the ML phylogenetic estimations, Best-fit ML models were chosen using MEGA version 5.02; Best-fit ML models were chosen as follows: GTR +  $\Gamma$  + I. In addition, phylogenetic analyses were performed using Bayesian approaches in MrBayes v3.1 (Huelsenbeck & Ronquist 2001). The program Kakusan4 (Tanabe 2007) was used to select appropriate models based on Schwarz's Bayesian Information (BIC) and the selected sites model: HKY85 +

Γ. Bayesian MCMC simulations were run for 100 million generations, sampling every 2,000 generations. The resulting tree was visualized in FigTree v1.3.1 (Rambaut 2009).

## Results

**Phylogenetic analysis.** Based on our genetic analysis of *S. formosana* and *S. drakon*, it was clearly revealed that they form two separate monophyletic clades (Clade I vs II; Fig. 1). This trend was supported as robust results of strong reliability in all analyzed methods (i.e., ML, NJ, and Bayesian analyses; Fig. 1). The Bayesian posterior probability support, NJ bootstrap support, and ML bootstrap support of each clade are 1.00/100/100, respectively (Fig. 1). From the positioning of the reference specimens, clade I corresponds to *S. formosana*, and clade II corresponds to *S. drakon*.



**FIGURE 1.** Bayesian phylogenetic relationships based on the sequence data of the mitochondrial DNA COI region (636-bp.) of *Stenopsyche* caddisflies. The numbers at major nodes indicate the Bayesian posterior probabilities, NJ, and ML bootstrap support, respectively. Numbers of the OTUs of clades I and II indicated specimen numbers in Table 1.

## Species description

### *Stenopsyche formosana* Kobayashi 1987

(Figs. 2, 3)

*Stenopsyche formosana* Kobayashi 1987, 37–38, 43, male, Taiwan; Malicky 2014, 1624, Taiwan.

*Stenopsyche banksi*: Mosely 1942 (in part), 359, Taiwan; Nishimura *et al.* 2015, 78, Taiwan.

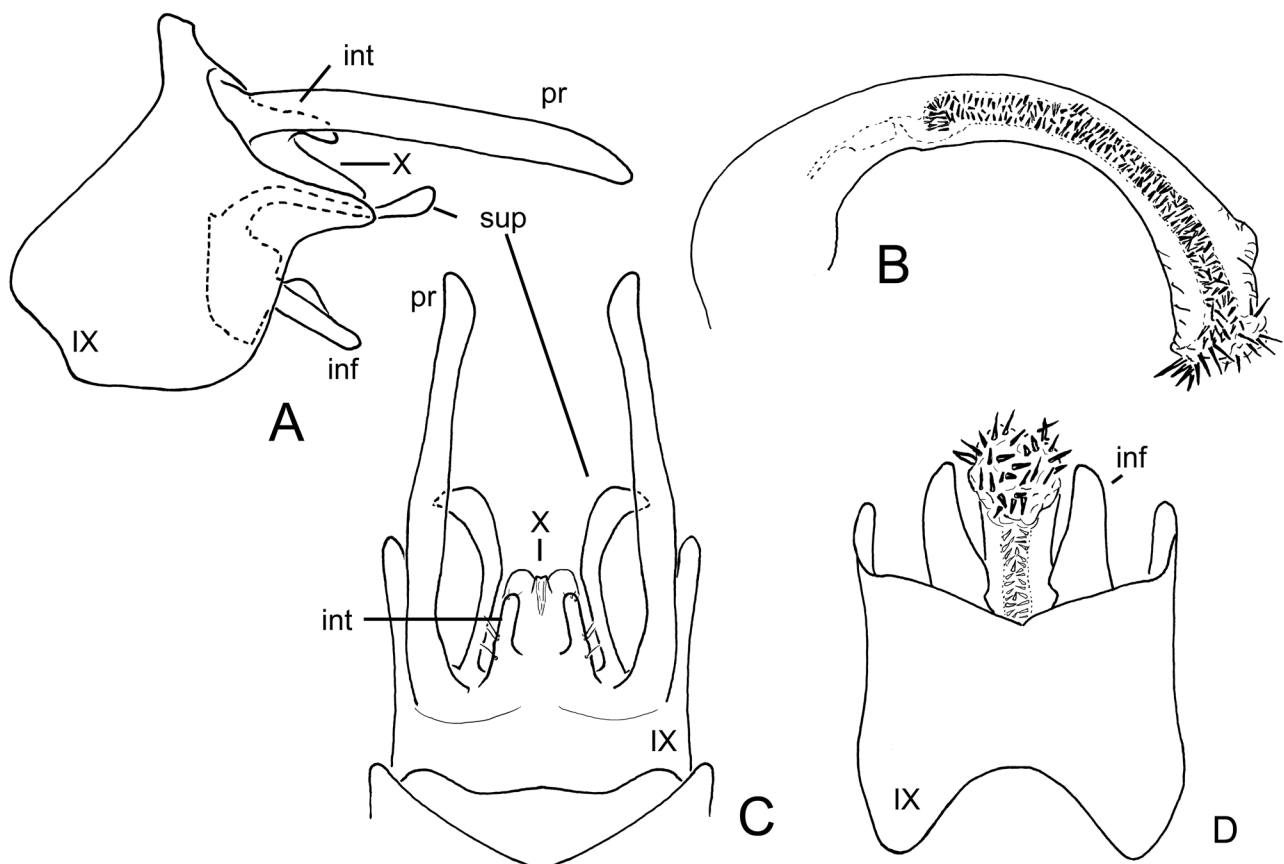
**Adult.** Forewings of male each 21–22 mm long (n=2) and of female 19–23 (n=3), brown with many pale spots, irregularly reticulated; hind wings white or slightly pale brown, subhyaline. Antennae more than 1.5 times as long as forewings in male, slightly longer than forewings in female. Legs yellowish brown, with dark markings on fore- and middle tibiae and on 1st to 2nd (often 3rd) segments of middle tarsi. Tibial spurs 3, 4, 4.

**Male genitalia.** Segment IX (IX) short dorsally, long ventrally, anteroventral margins round in lateral aspect, posterolateral margins each with long triangular projection in middle. Segment X (X) forming isosceles trapezoid

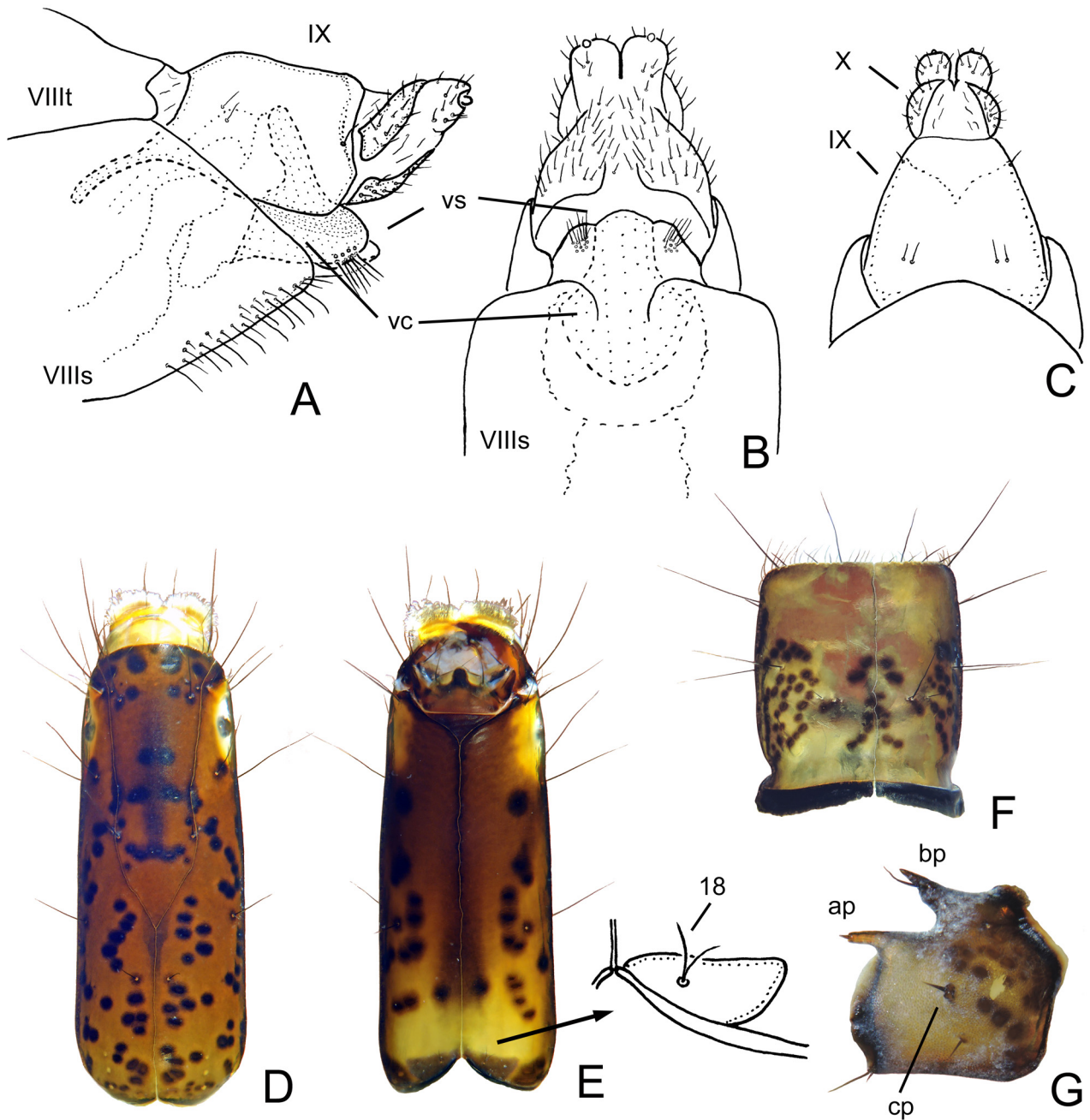
in dorsal aspect, semimembranous in apical half; apex slightly concave, with pair of stout lateral lobes. Pair of intermediate appendages (int) dorsolaterally near base, each with apex round, directed posterad. Preanal appendages (pr) long, more than 3 times as long as segment X. Inferior appendages (inf) long triangular in ventral aspect, each with round apex; superior arms (sup) about half as long as preanal appendages, evenly curved outward, apically semicircular in dorsal aspect. Phallus long, endotheca with numerous stout spines along its entire length.

**Female genitalia.** Segment IX (IX) rectangular in lateral aspect, mostly sclerotized but membranous posterodorsally and bearing 1 to 3 setae on each anterodorsal side, with long apodemal extension on each anteroventral corner. Segment X (X) (segment XI of Schmid 1969) membranous dorsally, with pair of setose longitudinal sclerites dorsolaterally; ventral setose area weakly sclerotized, but membranous apicomesally; with pair of setose distal lobes, each bearing cercus. Vulvar scale (vs) large, each side sclerotized, thumb-like in lateral aspect, with long stout setae apicolaterally; posterolateral margins round in ventral aspect, semimembranous mesally, projecting slightly beyond lateral corners. Vaginal chamber (vc) sclerotized, expanded anterad and dorsad in lateral aspect; ventral part wide, vase-shaped in ventral view.

**Final instar larva.** General appearance as in genus. Length up to 35 mm. Head 4.4–5.0 mm long (n=5); anterior part about 1.6 mm wide (eye level), posterior widest part about 1.8 mm; ground color yellowish to reddish brown, with many small dark spots; frontoclypeus with dark brown longitudinal band on mid-line, crossing three black transverse stripes; primary setae 1, 4, 10, 11, 13 not dark pigmented; setae 8, 15, 18 located ventrally; each seta 18 (18) bifurcated, about 0.2 mm long, located near postoccipital ridge, each surrounded by rugged semicircular area. Pronotum 2.3–2.5 mm long, about 2.3 mm wide; ground color yellowish brown, with many dark spots except for anterodorsal area, posterior margin black. Forecoxae pentagonal in mesal view; each apicodorsal process (ap) finger-like, straight, directed anterad, with short semitransparent spine-like seta apically; each basodorsal process (bp) elongate triangular, slightly longer than apicodorsal process, with long mesal seta in middle; each central process (cp) on mesal face small, with short stout seta apicodorsally.



**FIGURE 2.** Male genitalia of *Stenopsyche formosana*. A, left, lateral; B, phallus, left lateral; C, dorsal; D, ventral. Abbreviations: IX = segment IX, X = segment X, inf = inferior appendage, int = intermediate appendage, pr = preanal appendage, sup = superior arm of an inferior appendage.



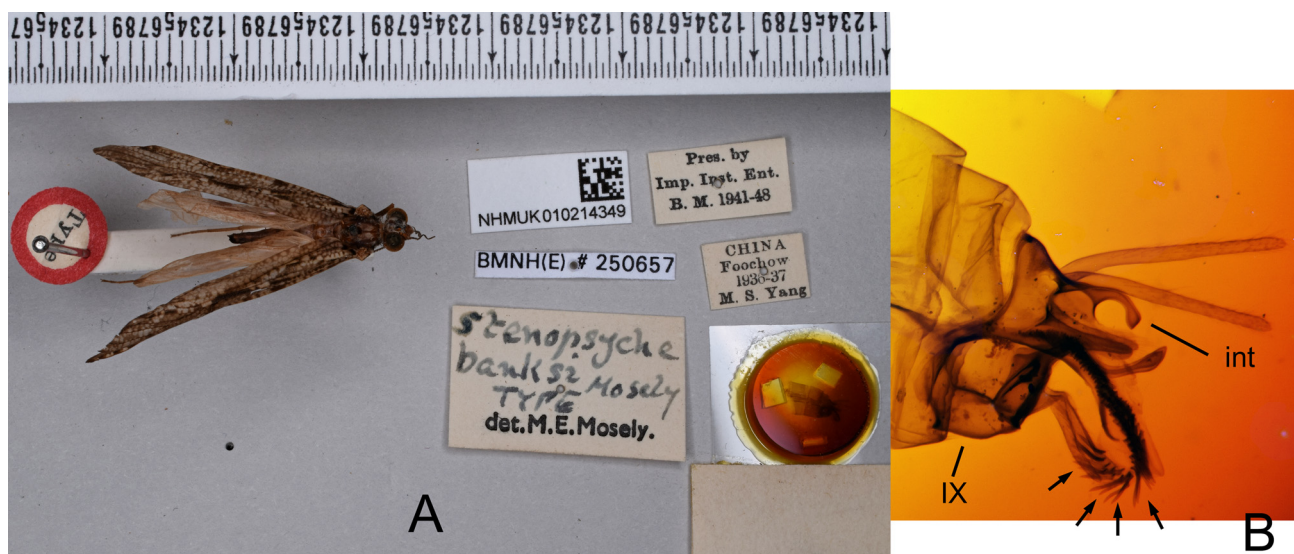
**FIGURE 3.** Female and larva of *Stenopsyche formosana*. A–C, female genitalia: A, left lateral; B, ventral; C, dorsal. D–G, larva: D, head, dorsal; E, head, ventral; F, prothorax, dorsal; G, mesal surface of right forecoxa, left lateral. Abbreviations: 18 = primary seta no. 18 of larval head, VIIIIs = sternum VIII, VIIIIt = tergum VIII, IX = segment IX, X = segment X, ap = apicodorsal process of a forecoxa, bp = basodorsal process of a forecoxa, cp = central process of mesal face of a forecoxa, vs = vulvar scale; vc = vaginal chamber.

**Specimens examined.** Holotype male (with handwritten label “7143 ♂”), [TAIWAN] Nangzunkei, wulia, 16.iv.1982, S. Uchida (CBM). [TAIWAN] Beigang River, Huisun Forest Area, Ren'ai Xiang, Nantou, 24°4.992'N, 121°0.651'E, alt. 490 m, 3.iii.2015, N. Nishimura, 1 male (SU); same locality, 3.iii.2015, L.P. Hsu, 1 male (TN); Huisun Forest Area, Ren'ai Xiang, Nantou, 24°5.628'N, 121°1.843'E, alt. 910 m, 30.iv.2010, K. Tojo, 2 larvae (SU); Meiyuan River, Ren'ai Xiang, Nantou, 24°5.09'N, 120°57.22'E, alt. 504 m, 3.iii.2015, N. Nishimura, 2 larvae (SU); same locality, 4.iii.2015, N. Nishimura, 3 females (1 female: SU, 2 females: TN); Sankeng Ecology Park, Dahan River, Longtan Xiang, Taoyuan, 24°50.169'N, 121°15.329'E, alt. 127 m, 26.xi.2010, K. Tojo, 1 larva (SU).

**Remarks.** We examined the holotype of *S. formosana* deposited in the CBM, and confirmed it to be identical with males used in this study. The type locality of this species was recorded as “Nangzunkei, wulia” by Kobayashi (1987). Although we could not understand the meaning of “wulia” but “Nangzunkei” must be a Japanese reading of a Chinese locality name “Nanshan stream.” The collector of this specimen visited this stream in Ren'ai Xiang, Nantou County on 16 April 1982 (Uchida personal communication).

On the other hand, Mosely (1942) described *S. banksi*, a species similar to *S. formosana*, from Foochow (Fuzhou, P.R. China: the holotype) in China, and also recorded it from Taiwan based on a male specimen designated as a paratype. *Stenopsyche banksi* has been recorded from north to central Taiwan (Biodiversity Research Center, Academia Sinica 2015). For confirming the taxonomic status of *S. formosana*, we compared males of *S. formosana* with photographs of the holotype and paratype males of *S. banksi* kindly provided by Dr. B. Price, The Natural History Museum, London. The paratype male of *S. banksi* from Taiwan is identical to our males of *S. formosana*. However the *S. banksi* holotype from continental China is slightly different from the paratype. Segment IX in lateral aspect is longer in *S. formosana* than that in the holotype of *S. banksi* (Figs. 2A, 4B); a pair of intermediate appendages is directed posterad in *S. formosana* (Fig. 2A) but they are large and strongly curved ventrad in the holotype of *S. banksi* (Fig. 4B; Mosely 1942, fig. 51); spines arising from the base of the endotheca are short in *S. formosana* (Figs. 2B, 2D), but very long in the *S. banksi* holotype (Fig. 4B; Mosely 1942, figs. 51, 53). Furthermore, in *S. formosana* a pair of intermediate appendages of the segment X arise from the dorsolateral sides of segment X (Fig. 2C), but they arise dorsally and close to each other in the *S. banksi* holotype (Mosely 1942, 358, fig. 52). Although *S. formosana* may be a subspecies level taxon of *S. banksi*, we treat them as distinct species. Further study with molecular data is needed to confirm the taxonomic status of this/these species.

The larva of this species is very similar to those of *S. angustata* Martynov 1930 (Hoang & Bae 2007, Xu *et al.* 2015) and *S. schmidi* Weaver 1987 (Tanida 2003, Nozaki & Shimura 2015), but is distinguishable from them by the shape or length of the apicodorsal processes of the forecoxae. In this species, the apicodorsal process of each forecoxa is straight and slightly shorter than the basodorsal process, but it is curved ventrad in *S. angustata* and less than half as long as the dorsal process in *S. schmidi*.



**FIGURE 4.** Holotype male of *Stenopsyche banksi* (Photos B. Price). A, body and genitalia (mounted), B, genitalia, left lateral. Abbreviations: IX = segment IX, int =intermediate appendage. Arrows indicate long spines arising on basal part of endotheca.

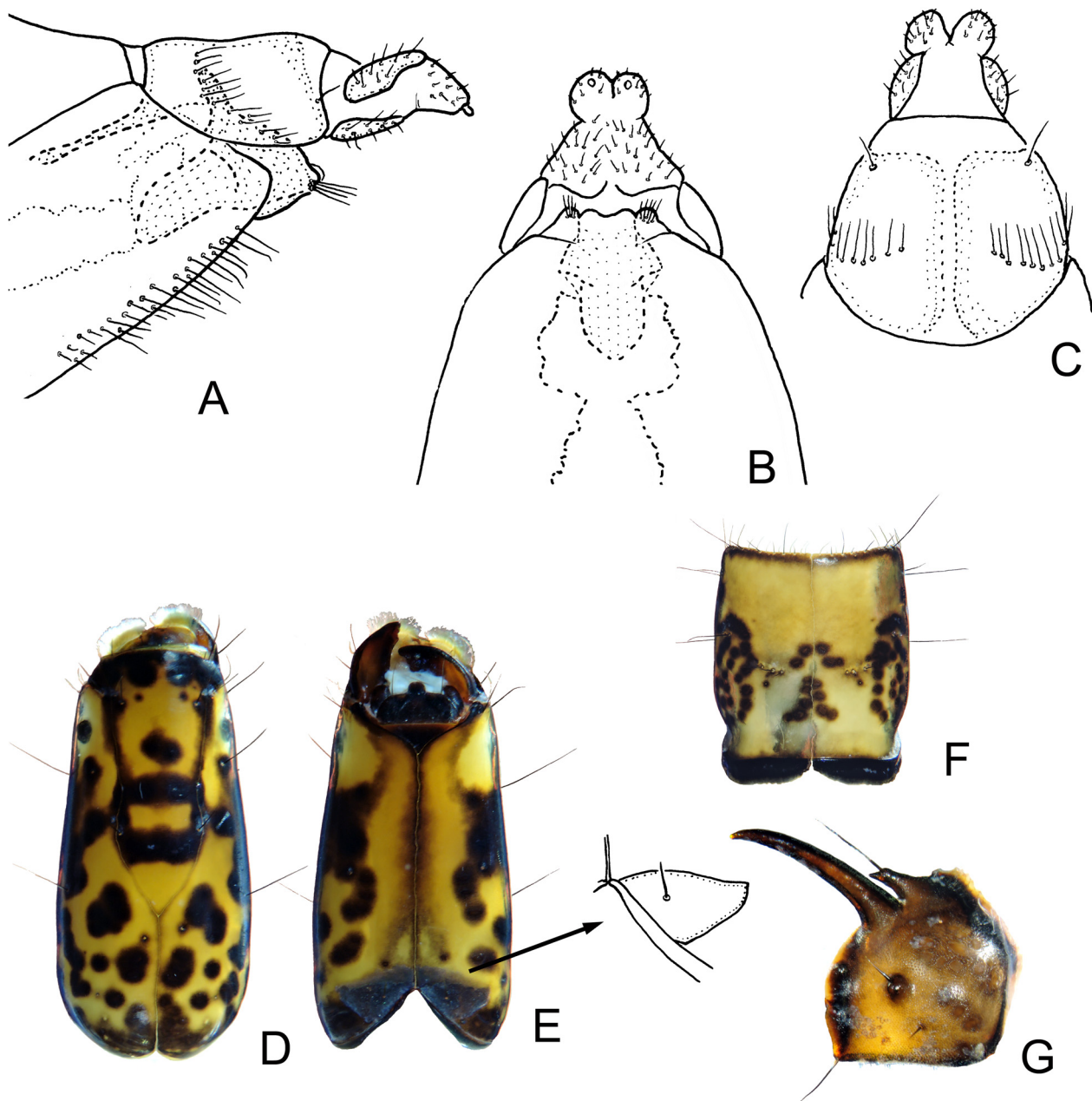
### *Stenopsyche drakon* Weaver 1987 (Fig. 5)

*Stenopsyche drakon* Weaver 1987, 165–167, male, Taiwan; Malicky 2014, 1624, Taiwan; Nishimura *et al.* 2015, 78, 81, Taiwan.

**Adult.** Forewings of male each 19 mm long (n=2), of female 20–22 (n=3), brown with many pale spots, irregularly



reticulated; hind wings white or slightly pale brown, subhyaline. Antennae about 1.5 times as long as forewings in male, slightly longer than forewings in female. Legs yellowish brown, middle legs with dark markings on tibiae and 1st to 2nd segments of tarsi, markings on foretibiae indistinct. Tibial spurs 3, 4, 4.



**FIGURE 5.** Female and larva of *Stenopsyche drakon*. A–C, female genitalia: A, left lateral; B, ventral; C, dorsal. D–G, larva: D, head, dorsal; E, head, ventral; F, prothorax, dorsal; G, mesal surface of right forecoxa, left lateral.

**Female genitalia.** Segment IX rectangular in lateral aspect, with long apodemal extension on each anteroventral corner; in dorsal view mostly sclerotized but separated by membrane along dorsal midline, posterodorsal area membranous, each sclerotized plate with transverse row of setae about 1/3rd distance from anterior margin, 1 pair of setae near posterior margin. Segment X (segment XI of Schmid 1969) membranous dorsally, with pair of setose longitudinal sclerites dorsolaterally; ventral setose area weakly sclerotized with apicomeral incision; apex divided into pair of distal lobes, each with cercus. Vulvar scale large; each side sclerotized, subrectangular in lateral aspect, with long stout setae apicolaterally; posterior margin sinuous in ventral aspect, membranous mesally. Vaginal chamber sclerotized, projecting anterodorsad in lateral aspect; ventral part pentagonal posteriorly in ventral aspect, tongue-shaped anteriorly in lateral and ventral aspects.

**Final instar larva.** Length up to 35 mm. Head 3.6–3.8 mm long (n=2); anterior part about 1.6 mm wide (eye level), posterior widest part about 1.8 mm; ground color yellowish brown, with many dark distinct spots and markings, frontoclypeus without dark longitudinal band on mid-line; with irregular dark spots and two small dots anteriorly, big dark spot and two transverse stripes on middle and posterior position; other parts of head with various dark spots; primary setae 1, 4, 10, 11, 13 not dark pigmented; setae 8, 15, 18 located ventrally; setae 18 not bifurcated, about 0.25 mm, located near postoccipital ridge, each surrounded by rugged, triangular area. Pronotum 2.4–2.6 mm long, about 2.2 mm wide, ground color yellowish brown, with many small dark spots except for anterodorsal area, posterior margin black. Forecoxae pentagonal in mesal view, each apicodorsal process very long, horn-like, directed anterad, curved slightly ventrad; each basodorsal process about 1/4 as long as apicodorsal process, close to apicodorsal process, with long dorsal seta near apex; each central process on mesal face with short stout seta dorsally in middle.

**Specimens examined.** [TAIWAN] Beigang River, Huisun Forest Area, Ren'ai Xiang, Nantou, 24°4.992'N, 121°0.651'E, alt. 490 m, 3.iii.2015, N. Nishimura, 1 male, 2 larvae (SU); same locality, 3.iii.2015, L.P. Hsu, 2 females (1 female: SU, 1 female: TN); Meiyuan River, Ren'ai Xiang, Nantou, 24°5.09'N, 120°57.22'E, alt. 504 m, 4.iii.2015, N. Nishimura, 1 male, 1 female (1 male: SU, 1 female: TN).

**Remarks.** The female of this species has dorsum IX mostly sclerotized but separated by membrane along the dorsal mid-line. A continental species, *S. huangshanensis* Xu *et al.* 2015, also has this character (Xu *et al.* 2015), but *S. huangshanensis* can be distinguishable from the former by the lack of a row of setae on each sclerotized plate. The larva of this species can be easily distinguished from known *Stenopsyche* larvae by the combination of following characteristics: Large distinct markings of the head but lacking dark longitudinal band on mid-line of frontoclypeus, unbifurcated no. 18 setae of the head, and long apicodorsal process of each forecoxa.

## Discussion

Xu *et al.* (2015) associated identifiable males of four Chinese *Stenopsyche* species with their females and larvae by mitochondrial DNA COI sequences. In this study, we also analyzed two *Stenopsyche* species collected from Taiwan, and their monophyly at the species level was strongly supported by the mitochondrial DNA COI region (Fig. 1). Thus, we were able to identify the species for *Stenopsyche* caddisflies using the DNA barcoding method. We suggest that the effectiveness of the DNA barcoding method is demonstrated clearly with regard to the *Stenopsyche* caddisflies.

Schmid (1969) and Weaver (1987) recognized six species groups in the genus *Stenopsyche* based on male genitalia, and Xu *et al.* (2014) assigned *S. formosana* to the *S. simplex* Group mainly based on the short segment X of the male genitalia illustrated by Kobayashi (1987). However, the *S. formosana* specimens examined in this study have segment X longer than the intermediate appendages (Figs. 2A, C), although segment X of the holotype male was over-cleared. The shape of segment X in dorsal view and the numerous spines dispersed along entire length of the endotheca in this species are rather similar to those of males belonging to the *S. marmorata* Group. The larva of *S. formosana* is also similar to those of *S. angustata* Martynov 1930 (Hoang & Bae 2007, Xu *et al.* 2015) and *S. schmidi* Weaver 1987 (Tanida 2003, Nozaki & Shimura 2015), both belonging to the *S. marmorata* Group as indicated by the presence of the dark longitudinal band on the mid-line of the frontoclypeus, the shape and length of no. 18 setae on the head, and the shape of the forecoxae (Figs. 3D, E, G).

On the other hand, Weaver (1987) assigned *S. drakon* to the *S. marmorata* Group, despite the fact that the larva is very different from known larvae of the *S. marmorata* Group in the large dark markings and unbifurcated no. 18 setae on the head, and the shape of the forecoxae. Further studies including molecular study are needed to solve the phylogenetic relationships in the genus *Stenopsyche*.

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providing the information about the collecting site of the holotype of *S. formosana*. We are also indebted to K. Hatta, Nagoya Women's University; staff of Entomological Department of the National Chung Hsing University; A.T-S. Lin, the National Central University, Taiwan, for their cooperation with the field research and collection of specimens in Taiwan. Finally, we are deeply grateful to C.H. Sun, Nanjin Agriculture University, J.C. Morse, Clemson University, and an anonymous reviewer for their valuable suggestion to improve the manuscript.

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