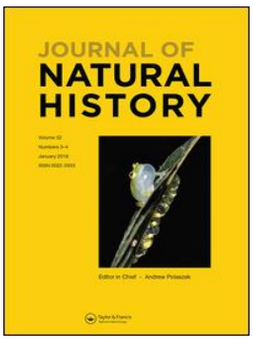

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New damselfly hosts and species identification of an aquatic parasitoid *Hydrophylita emporos* (Hymenoptera: Trichogrammatidae) in Taiwan

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ABSTRACT

The host–parasitoid relationship and species identity of aquatic parasitoids of two damselflies *Coeliccia cyanomelas* (Platycnemididae) and *Psolodesmus mandarinus dorothea* (Calopterygidae) from Fushan and Lienhuachih in Taiwan were studied using morphological characters and DNA barcoding sequences. The parasitoids reared from the damselflies' eggs, and the field-collected parasitoids, were morphologically identified as *Hydrophylita emporos* (Trichogrammatidae), a recently described parasitoid of the damselfly *P. m. mandarinus* from Northern Taiwan. The *CO1* (cytochrome c oxidase I) gene tree supported the identification as *H. emporos*, as well as all parasitoid samples from *C. cyanomelas, P. m. dorothea* and *P. m. mandarinus*. The sampled *H. emporos* populations did not differ genetically despite their different host associations. However, some genetic differences were found between *H. emporos* populations from Northern and Central Taiwan, indicating that the dispersal of *H. emporos* may be limited by geographical distances. Our results suggest that *H. emporos* can parasitise not only closely related sister subspecies, *P. m. mandarinus* and *P. m. dorothea*, but also phylogenetically distant species of another damselfly family, *C. cyanomelas*. This is the first record of multiple damselfly hosts for the aquatic parasitoid genus *Hydrophylita*. This finding implies that the host range of *H. emporos* and congeneric species may be broader than previously thought.

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Coeliccia cyanomelas; *CO1*; DNA barcode; Odonata; *Psolodesmus mandarinus*; taxonomy

Introduction

Hydrophylita Ghesquière 1946 (Hymenoptera, Trichogrammatidae) is a small genus of partly aquatic parasitoid wasps, currently with five described species, all known to parasitise eggs of damselflies (Odonata, Zygoptera) (Pinto 2006; Querino and Pinto 2007; Shih et al. 2013). Available host records for *Hydrophylita* species indicate that they are parasitoids of at least three damselfly families: Calopterygidae (*Psolodesmus mandarinus): H. emporos* from Taiwan, Asia (Shih et al. 2013); Coenagrionidae (*Ischnura verticalis): H. aquivolans* from North America (Matheson and Crosby 1912; Davis 1962); and Lestidae (*Lestes* sp.): *H. lestesi* from Brazil, South America (Costa Lima 1960). The damselfly

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hosts for *H. bachmanni* from Argentina (De Santis 1964) and *H. neusae*, widespread in Neotropics (Querino and Pinto 2007), are not known. However, little is known about the host range of *Hydrophylita* species and whether they are host specific.

Hydrophylita emporos Shih & Polaszek is a recently described parasitoid species of *P. mandarinus* from Shimen in Northern Taiwan (Shih et al. 2013). Females of this parasitoid were found to be phoretic on the base of the abdomens of female *P. mandarinus*. When the damselfly starts laying eggs under the water, the parasitoid walks along the abdomen towards its tip to parasitise the newly laid damselfly eggs in the plant tissue (Shih et al. 2013; https://vimeo.com/59398646). In Taiwan, there are two subspecies of *P. mandarinus*: *P. m. mandarinus* McLachlan from Northern Taiwan and *P. m. dorothea* Williamson from Central and Southern Taiwan (Lin et al. 2014). An earlier study found no infestation of *H. emporos* in *P. m. dorothea* populations in Central and Southern Taiwan (Shih et al. 2013), even though these two subspecies share ecologically similar habitats of small, fast running streams in the lowland forests. However, our field observations of the parasitoids of *Coeliccia cyanomelas* (Platycnemididae) and *P. m. dorothea* from Lienhuachih in Central Taiwan suggested the probable existence of *Hydrophylita* in these two damselflies (Figure 1(a–c)). Because parasitoid species frequently diverge genetically or speciate via host-shifting (e.g. Hamerlinck et al. 2016), these observations raised the question whether the parasitoids of the distantly related *C. cyanomelas* and those of the two closely related *P. mandarinus* subspecies are the same species, *H. emporos*.

This study aims to examine the host–parasitoid relationship of the parasitoids of *C. cyanomelas* and *P. m. dorothea* populations using field observations and by rearing parasitised eggs of the damselflies. Species identity of the parasitoids in these damselflies was investigated by analysing morphological characters and DNA barcoding sequences.

Materials and methods

Insect collecting and rearing

Field collecting of the parasitoids of the damselflies was carried out by manually searching for damselflies at forest streams in Fushan, Yilan County of Northern Taiwan and in Lienhuachih, Nantou County of Central Taiwan each summer (July to September) from 2015 to 2017 (Figure 1(c)). There was a total of 188 and 143 search days in Fushan and Lienhuachih, respectively. To confirm the damselfly host species of the parasitoids, the ovipositional behaviour of the damselfly hosts and the associated parasitoids was observed. When the mating and oviposition of the damselflies were found, we started searching for the parasitoids on the damselfly's bodies, in the vicinity of the damselflies, and on the ovipositional substrates of the damselflies, until the departure of the mating pairs or ovipositing females. Adult parasitoids were manually collected using an insect pin with a droplet of 95% ethanol on its tip. The collected parasitoid specimens were preserved in 95% ethanol immediately after capture.

The ovipositional substrates and deposited eggs of the damselfly females associated with the parasitoids were brought back to the laboratory to rear the parasitoids. In the laboratory, the damselfly eggs were removed from the substrates and then transferred onto a filter paper (ADVANTEC, Tokyo, Japan) immersed in distilled water to a depth less

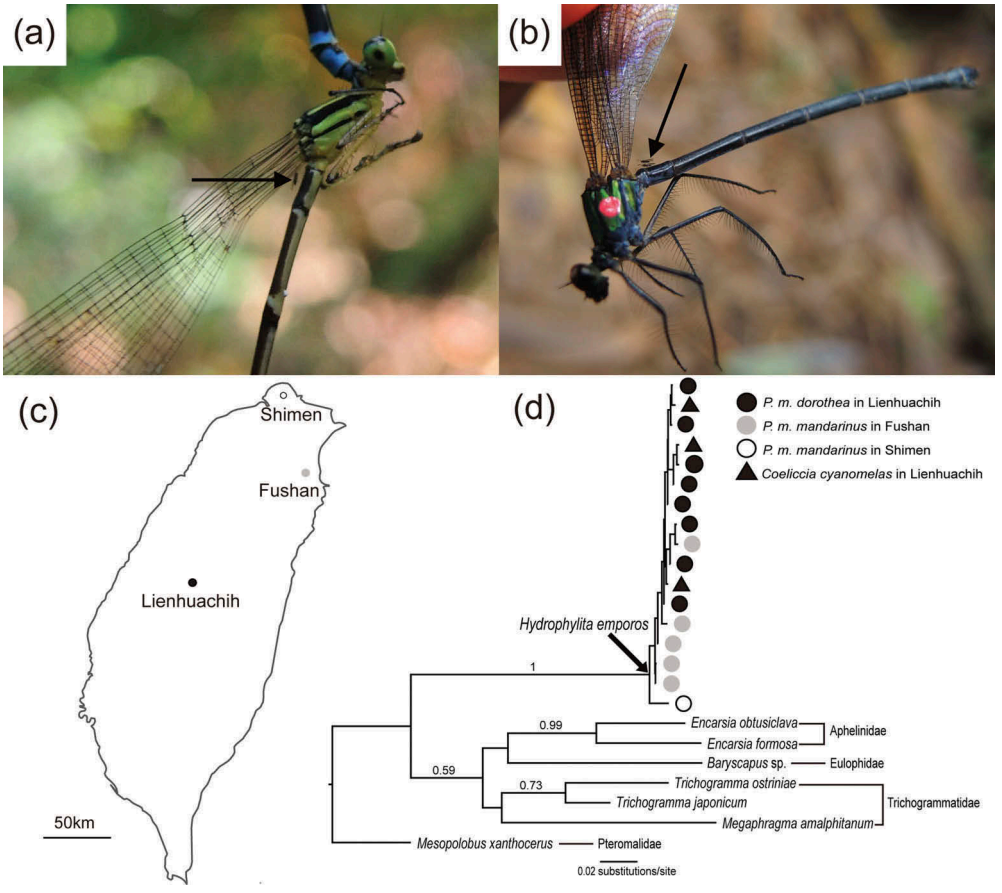

Figure 1. *Hydrophylita emporos* stands near the base of the abdomen of damselflies (a) *Coeliccia cyanomelas* and (b) *Psolodesmus mandarinus dorothea*. (c) Map of Taiwan shows the type locality (Shimen) of *Hydrophylita emporos* and the two field sites (Fushan & Lienhuachih) in this study. (d) *CO1* gene tree of *Hydrophylita emporos* samples collected from *Coeliccia cyanomelas, Psolodesmus mandarinus dorothea* and *Psolodesmus mandarinus mandarinus*. Numbers above the branches are support values of the Bayesian Posterior Probability (BPP). Branches without support values have values <50%.

than three times the egg's height in a 35 × 15 mm plastic Petri dish (Alpha Plus, Taiwan). Each Petri dish was placed in the laboratory at a temperature of about 25°C and a photoperiod of 13 h:11 h (light:dark). The damselfly eggs were photographed every 5–10 days to record the morphological changes, using a digital camera (EOS700D, Canon, Tokyo, Japan) mounted on a stereomicroscope (SZ61, Olympus, Tokyo, Japan) at magnifications of 10–45×. The emerged parasitoid adults were observed for their mating behaviour until they died. They were then preserved in 95% ethanol in a −20°C freezer.

Specimen preparation, identification and scanning electron microscope

The parasitoid specimens were mounted in Canada balsam and Euparal following a protocol modified from Noyes (1982). The specimens preserved in ethanol were first cleared in 10% KOH, dehydrated through graded 45–100% alcohol and then mounted in

Canada balsam (with Hinoki oil) or Euparal. The species identification of the specimens was confirmed using the morphological description and key of Shih et al. 2013 and by comparison with the five paratypes of *H. emporos* (four females and one male) in the insect museum of the National Taiwan University, Taipei, Taiwan. The slide-mounted specimens were examined and photographed using a digital camera (α6000, Sony, Tokyo, Japan) mounted on a light microscope Axiolab E re (Carl Zeiss, Oberkochen, Germany). Six specimens were dehydrated and coated with gold for the scanning electron microscope (SEM) (JSM-5610, JEOL, Tokyo, Japan). Morphological terminology associated with the antenna sensilla and proprioceptor follows those used for *Trichogramma* (Pinto 2006) and *Pseudoligosita yasumatsui* (Trichogrammatidae) (Wong et al. 2019), respectively, including trichoid sensilla type 5 (Tri. 5), aporous seta A (APA), aporous sensillar trichodea B (socketed) (APB), coeloconic sensilla (CS), basiconic peg sensilla (BPS), flagelliform setae or multiporous pitted sensilla trichodea A (unsocketed) (FS), placoid sensilla (PLS), recurved sensilla (RS) and uniporous pit pore sensilla trichodea D (UPP).

DNA barcoding and phylogenetic analyses

Genomic DNA of the parasitoids was extracted using two protocols. The first protocol used REPLI-g® Single Cell Kit (QIAGEN, GmbH, Hilden, Germany) for DNA extraction and genome amplification following the manufacturer's instructions. The second protocol was a Chelex method (Bruzzese 2016). The specimen preserved in 95% ethanol was removed and placed in a 1.5-ml eppendorf tube until all ethanol was evaporated. Then, 10 µl of proteinase K (10 mg/ml) and 150 µl of 10% Chelex®100 resin were added, and the specimen was ground using sterilised pipette tips. The solution was incubated and stirred periodically at 55°C for 24 h. The tube was spun to pellet Chelex resin and suspend the DNA in the top clear layer of the solution.

The DNA barcoding fragment of mitochondrial *CO1* (cytochrome c oxidase I) gene was amplified using FCO and RCO primers (Shih et al. 2013). The 70 µl polymerase chain reaction (PCR) contained 1.4 µl Super-Run Ex *Taq* DNA Polymerase (2 u/µl, Protech Technology, Taiwan), 2.8 µl of 10 mM of each primer, 5.6 µl of 25 mM dNTPs, 7 µl of Super-Run Ex *Taq* 10× buffer, 44.8 µl of ddH2O and 5.6 µl of genomic DNA. The PCR was carried out in a thermocycler (Biometra TOne, Analytik, Jena, Germany) with the following protocol: (i) an initial denaturation at 94°C for 5 min; followed by (ii) 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min; and (iii) a final extension step at 72°C for 10 min. The PCR products were purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan). The purified PCR products were either sequenced directly or cloned into vectors (DH5α, Protech Technology, Taiwan) using the RBC TA Cloning Vector Kit (RBC Bioscience, Taipei, Taiwan). The M13R and M13F primers were used to amplify positive clones to confirm the lengths of target fragments. DNA sequencing of purified PCR products and cloned fragments was performed on an ABI 3730xl DNA Analyser (Perkin Elmer, CA, USA) at the Genome Research Centre of National Yang-Ming University (Taipei, Taiwan).

The *CO1* sequences were manually edited using SeqMan in DNASTAR (LASERGENE, Swindell and Plasterer 1997) and translated into amino acid sequences using an invertebrate mitochondrial genetic code in Mesquite v.3.51 (Maddison and Maddison 2018) to check for possible stop codons caused by ambiguous sequencing. The *CO1* sequence of

H. emporos (KF053530) from the type locality (Shimen, Northern Taiwan, Shih et al. 2013) and the available closely related outgroup species were downloaded from GenBank [*Trichogramma japonicum* (NC039534), *Trichogramma ostriniae* (NC039535), *Megaphragma amalphitanum* (KT373787), *Encarsia obtusiclava* (MG813798), *Encarsia formosa* (MG813797), *Baryscapus* sp. (HM573858) and *Mesopolobus xanthocerus* (JQ416836)] and then aligned using the Clustal W method in MegAlign (DNAStar package, Madison, USA).

Pairwise sequence divergence between the *CO1* sequences was calculated using a maximum composite likelihood model in MEGA7 (Kumar et al. 2016). Phylogenetic analyses were performed using the Bayesian method. The best-fitting nucleotide substitution model for the Bayesian analyses was selected in jModelTest v. 2.1.10 (Darriba et al. 2012) using the Bayesian Information Criterion: F81+I for codon position 1 and 2 and HKY+G for codon position 3 of *CO1*. Bayesian phylogenetic analyses were conducted in MrBayes v. 3.2.6 (Ronquist et al. 2012) using the Markov Chain Monte Carlo searches for 10^7 generations with a sampling frequency of every 1000 generations and a burn-in of 2.5 $\times 10^6$ generations. The Bayesian posterior probability of the tree branches was calculated from a 50% majority rule tree after discarding a burn-in of 25% of the sampled trees. The Bayesian analyses were carried out twice, firstly with unrooted trees and secondly with the most distant taxon (*Mesopolobus xanthocerus*) as an outgroup.

Results

Species identification and revised key to Hydrophylita *species*

A total of 30 females of the parasitoids of *P. m. dorothea* from Lienhuachih; 14 females and 3 males of the parasitoids of *C. cyanomelas* from Lienhuachih; and 5 females of the parasitoids of *P. m. mandarinus* from Fushan were slide-mounted and examined. The morphologies of all collected parasitoid specimens were in agreement with the description of *H. emporos* in Shih et al. 2013, except for the morphological variation of antennal sensilla. A re-description of the antennal sensilla of *H. emporos* is provided below, with the original descriptions (Shih et al. 2013) in the parentheses:

Female: antenna with 8 antennomeres. Radicle with 8–12 Tri. 5 (Figure 2(a)) (5 APA); Scape with 5 APB (3APA); Pedicel with 5 APB, 1 recurved trichodea sensillum (5APB) (Figure 2(b,c): RTS); A1 with 1 APB (1 APB); A2 with 1 CS (Figure 2(d)) (0); F1 with 3–5 APA or APB, 2 BPS (3–5 APB, 2 BPS); F2 with 4–6 APA or APB, 2 BPS (5–8 APA or APB, 2 BPS, 1FS); C1 with 4–6 APA or APB, 2 FS, 1 BPS, 1 PLS (4–7 APA or APB, 2 FS, 1–2 BPS, 1 PLS); C2 with 11–20 APA or APB, 1 FS, 2 BPS, 6 PLS, 2 RS (Figure 2(e,f)) (11–14 APA or APB, 1 FS, 1–2 BPS, 5 PLS, 3 subapical conelike sensilla, 2 spinelike RS, UPP at very apex of antennomere).

Male: antenna with 9 antennomeres. Radicle with 10 Tri. 5 (0); Scape with 5 APB (2 APA); Pedicel with 5 APB, 1 recurved trichodea sensillum (4 APB); A1 with 1 APB (0); A2 with 1 CS (0); F1 with 8–11 FS, 2 BPS (8 FS, 2BPS); F2 with 8–12 FS, 2 BPS (6 FS, 2 BPS); C1 with 8–11 FS, 1 BPS (7 FS, 1 BPS, 0 PLS); C2 with 5–7 FS, 1 PLS, 1 BPS (5 FS, 1 PLS, 1BPS); C3 with 4–6 FS, 2 PLS, 1 BPS (5 FS, 2 PLS, 1 BPS).

An error was found in the key to *Hydrophylita* species in Shih et al. 2013. The description of key couplet 3 to the species of female *Hydrophylita* did not match the morphology observed in our collected parasitoid specimens and the four determined paratypes of *H. emporos*. Couplet 3 states mistakenly 7 antennomeres for *H. emporos* and 8 antennomeres for *H. lestesi* and

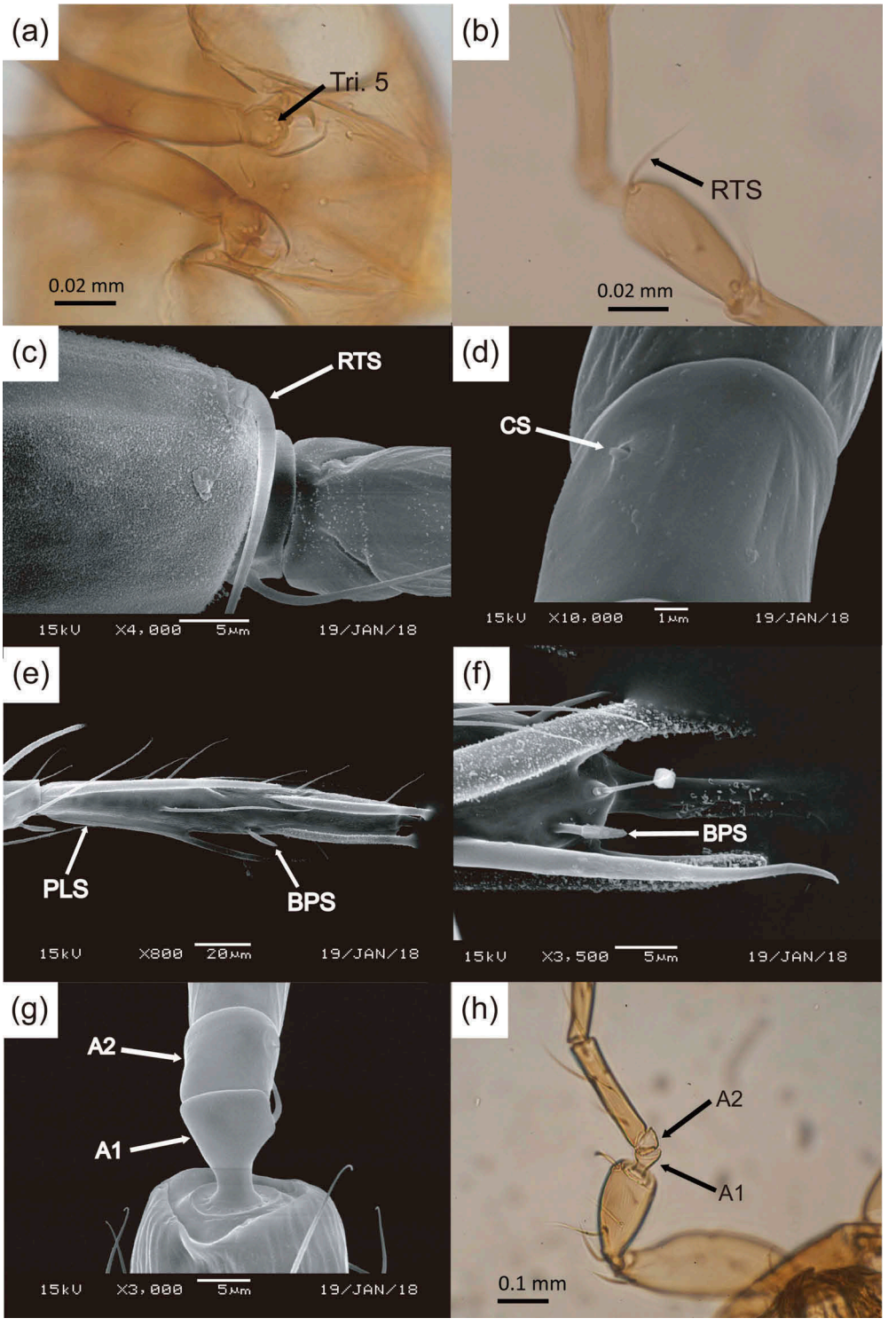

Figure 2. The antenna of female *Hydrophylita emporos*. (a) Radicle under light microscope. Arrow indicates the trichoid sensilla type 5 (Tri. 5). (b) Pedicel under light microscope. Arrow indicates the recurved trichodea sensillum (RTS). (c) Apex of the pedicel under SEM. (d) Anellus 2 under SEM. Arrow indicates the coeloconic sensilla (CS). (e) Club 2 under SEM. Arrows show the basiconic peg sensilla (BPS) and placoid sensilla (PLS). (f) Apex of C2. Arrow indicates a BPS at the apex of C2. (g) Anelli under SEM. (h) Anelli under light microscope. Arrows indicate anellus 1 (A1) and 2 (A2).

H. neusae. The correct number of antennomeres should be 8 for *H. emporos* and 9 for *H. lestesi*/*H. neusae*, respectively. This error has occurred by our including the anellus 1 (A1) segment of antenna in the antennomere count. Under the SEM, A1 segment of *H. emporos* was fused with pedicel (Figure 2(g)). A coeloconic sensillum (CS) was found on the next segment, so it was determined to be A2 (Pinto 2006). Under a light microscope, CS on A2 was too small to be clearly observed, but the anelli could still be identified as two separate segments (Figure 2(h)). Here, a revised key to female *Hydrophylita* species based on Shih et al. 2013 is provided (bold numbers indicate the revised key couplet 3):

1. Fore wing very narrow with apex distinctly pointed; its length at least 14× its width, disk with only a single line of setae. Antenna with placoid sensilla (PLS) on each claval antennomere attached to surface almost their entire length subgenus *Hydrophylita*.2
1′ Fore wing wider, with apex slightly pointed; its length less than 10× its width, disk densely setose. Antenna with one or more PLS on each claval antennomere spinelike, attached to surface only at the base ..... subgenus *Lutzimicron*.3
2. Antenna with one anellus (North America) . . .. *H. aquivolans*
2′ Antenna with two anelli (Central and South America) ..... *H. bachmanni*
3. Antenna with **8** antennomeres, C2 and C3 completely fused. (Asia) .... *H. emporos*
3′ Antenna with **9** antennomeres, C2 and C3 separate 4
4. Antenna with funicular antennomeres approximately equal in length, first claval antennomere (C1) approximately equal in length to first funicular antennomere (F1); clava poorly differentiated from funicle. Fore wing 8× as long as wide (Brazil) ... . *H. lestesi*
4′ Antenna with F2 and C1 both distinctly shorter than F1, clava well differentiated from funicle. Fore wing 6× as long as wide (broadly distributed in Neotropics) . . . *H. neusae*

CO1 sequence variation and gene tree

A total of 580 bp of mitochondrial *CO1* sequences was obtained from 16 parasitoids, including 8 individuals from *P. m. dorothea* from Lienhuachih; 3 individuals from *C. cyanomelas* from Lienhuachih; and 5 individuals from *P. m. mandarinus* from Fushan (GenBank accession numbers, MN266217–MN266232). The pairwise sequence divergence of *CO1* was negligible (0–0.5%) between these parasitoid specimens. In contrast, the sequence divergence of *CO1* was greater (1.1–1.4%) between these parasitoid specimens and *H. emporos* from the type locality in Shimen. The *CO1* gene tree strongly supported a monophyletic lineage of all parasitoid specimens and *H. emporos*, indicating that they belong to the same phylogenetic species (Figure 1(d)).

Damselfly hosts of H. emporos

Hydrophylita emporos was found to parasitise three damselfly species, *C. cyanomelas* in Lienhuachih ($n = 34$), *P. m. mandarinus* in Fushan ($n = 23$) and *P. m. dorothea* in Lienhuachih ($n = 255$) (Figure 1(a,b)). In 2016 at Lienhuachih, *H. emporos* was found on 93 *P. m. dorothea* individuals, in which 51 were ovipositing (51/93 = 54.8%), 15 were mating (15/93 = 16.1%) and 27 were conducting non-reproductive behaviours (27/93 = 29.0%). One clutch of 120 *C. cyanomelas* eggs on a fallen leaf was collected and reared in the laboratory. A total of 15

Figure 3. The morphology of the eggs of *Coeliccia cyanomelas* parasitised by *Hydrophylita emporos* in 17, 26 and 30 days. The arrow indicates the embryo of immature *Hydrophylita emporos*.

eggs were parasitised (15/120 = 12.5%) by *H. emporos*. Under laboratory condition of 25°C and a photoperiod of 13 h:11 h (light:dark), the embryo of immature *H. emporos* was visible in the parasitised eggs after approximately 17 days (Figure 3). The outline of adult *H. emporos* appeared after nearly 26 days. Adult *H. emporos* emerged by biting a hole in the host egg. Six females and six males (sex ratio = 1) of *H. emporos* successfully emerged (12/15 = 80%) from the parasitised eggs in about 30 days (29.6 ± 0.7 days, n = 12). Unfortunately, no mating behaviour of these emerged adults was observed in the laboratory.

Discussion

The morphological characters and *CO1* gene tree together clearly demonstrate that all collected and examined parasitoids are *H. emporos*. With the caveat of short branches of in-group lineages, thus weak nodal supports, the *CO1* haplotypes of *H. emporos* were not clustered by damselfly hosts (*C. cyanomelas, P. m. mandarinus* & *P. m. dorothea*) (Figure 1(d)), indicating that these *H. emporos* populations did not differentiate genetically through their host associations. However, the *CO1* haplotypes of *H. emporos* showed basal Northern Taiwan (grey, Shimen & Fushan) vs. derived Central Taiwan (black, Lienhuachih) lineages except for a potential recent migrant/gene flow of *H. emporos* in *P. m. mandarinus* from Fushan (Figure 1(d)), suggesting some genetic differentiation between *H. emporos* populations of Northern and Central Taiwan, and that the dispersal of aquatic parasitoids like *H. emporos* can be limited by geographical distances. The *CO1* haplotype of *H. emporos* from the type locality of Shimen showed the greatest genetic divergence from all the other *H. emporos* haplotypes from Fushan and Lienhuachih,

suggesting that either it represents an ancestral haplotype, or *H. emporos* from Shimen is genetically isolated from the other *H. emporos* populations.

This study discovered that *C. cyanomelas* (Platycnemididae) and *P. m. dorothea* (Calopterygidae) are damselfly hosts of *H. emporos*, in addition to the originally described host *P. m. mandarinus*. To our knowledge, this is the first record of more than one damselfly host for any species in the aquatic parasitoid genus *Hydrophylita*. Our results suggest that *H. emporos* can parasitise not only closely related sister subspecies, *P. m. mandarinus* and *P. m. dorothea*, but also a phylogenetically distant species, *C. cyanomelas* of another damselfly family Platycnemididae. Ecologically similar egg-laying behaviour shared by *P. mandarinus* and *C. cyanomelas* in small forest streams may facilitate the parasitism of *H. emporos*. Females of both *P. mandarinus* and *C. cyanomelas* spend a long time probing below the surface of the water to lay eggs on submerged plants. The parasitoids were observed to position themselves on the abdomens of the damselflies waiting for their oviposition. Therefore, the abdomen of female damselfly conducting ovipositional behaviour effectively acts as a bridge connecting *H. emporos* females and the newly laid eggs, overcoming the potential difficulty of the small parasitoid breaking the surface tension of the water (Davis 1962; Shih et al. 2013). Similar ovipositional behaviour has been found in other damselfly hosts of *Hydrophylita* (e.g. *Ischnura verticallis*, Fincke 1987).

Our findings also suggest that the host range of *H. emporos*, and probably other congeneric species, may be broader than previously thought. For the other two aquatic parasitoids of the Trichogrammatidae, *Centrobiopsis* and *Prestwichia* (Bennett 2007), available records suggest that they parasitise multiple insect hosts [3 damselflies, *Lestes dryas, L. uncatus* & *L. unguiculatus* (Lestidae) in *C. odonatae*; 6 beetles, *Acilius* sp., *Agabus* sp., *Cybister* sp., *Dytiscus* sp., *D. marginalis* (Dytiscidae) & *Pelobius* sp. (Hygrobidae); 3 bugs, *Aphelocheirus montandoni* (Aphelocheiridae), *Ranatra linearis* (Nepidae) & *Notonecta* sp. (Notonectidae); 3 odonates, *Aeschna* sp. (Aeschnidae), *Agrion* sp. (Coenagrionidae) & *Lestes* sp. (Lestidae) in *Pr. aquatica*; 2 bugs, *Anisops bouvieri* (Notonectidae) & *Plea frontalis* (Pleidae) in *Pr. indica*; 3 odonates, *Aeschna* sp. (Aeschnidae), *Agrion* sp. (Coenagrionidae) & *Lestes* sp. (Lestidae) in *Pr. solitaria*] (Noyes 2019). This study also extends the geographical distribution of *H. emporos* from the type locality of Shimen to include the neighbouring Fushan of Northern Taiwan and Lienhuachih of Central Taiwan. However, our studies of *H. emporos*'s hosts were limited to summer seasons (July to September) and with only a few sampling populations. Further studies to investigate the host range and specificity of *Hydrophylita* are needed by including larger samples, additional sites and potential hosts and non-summer seasons.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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