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Taxonomy and biology of a new ambrosia gall midge *Daphnephila urnicola* sp. nov. (Diptera: Cecidomyiidae) inducing urn-shaped leaf galls on two species of *Machilus* (Lauraceae) in Taiwan

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Abstract

Recent field surveys show that galls induced by *Daphnephila* spp. (Cecidomyiidae) on *Machilus* spp. (Lauraceae) are common in Taiwan, yet only five species, four leaf-gall inducers and one stem-gall inducer on *M. thunbergii,* have been named in the past. Here we describe a new species, *Daphnephila urnicola* **sp. nov.** Chiang, Yang & Tokuda, inducing urn-shaped galls on leaves of both *M. zuihoensis* and *M. mushaensis*. Comparisons of *D. urnicola* populations on *M. zuihoensis* and on *M. mushaensis*, indicate that they belong to one species, a result supported by gall midge morphology, life-history traits, gall shape and structure, the developmental process of gall tissues, fungal associations, and DNA-sequencing data. Size and structure of the gall operculum was found to differ between *M. zuihoensis* and *M. mushahaensis*.

Key words: fungus, gall tissue, life cycle, mycangia, parasitoid

Introduction

Daphnephila Kieffer (Diptera: Cecidomyiidae) species in Taiwan induce at least 43 different sorts of galls, which can be placed into eight groups based on gall morphology, on eight species of *Machilus* (Lauraceae) (Yang & Tung 1998; Tung *et al*. 2006; Chiang & Yang unpublished data). This is currently considered to be the most diverse genus of gall-inducing insects in Taiwan (Yang & Tung 1998; Tung *et al*. 2006). However, only five of the species have been described, all from *Machilus thunbergii* Sieb & Zucc., including four leaf- and one stem-gall inducing taxa (Yang & Tung 1998; Tung *et al*. 2006; Tokuda *et al.* 2008; Gagné & Jaschhof 2014).

Daphnephila belongs to Asphondyliini, which usually induce 'ambrosia galls' with fungal hyphae growing around the inside of larval chambers within galls (Bissett & Borkent 1988; Bronner 1992; Carroll 1992; Rohfritsch 2008). Galls induced by species of *Daphnephila* on the leaves of *M. thunbergii* in Taiwan develop a well-defined nutritive tissue with fungal hyphae occurring among the cells of those tissues (Chen & Yang 2008; Chao & Liao 2013). The commonly occurring fungus in such ambrosia galls is *Botryosphaeria dothidea* (Ascomycota: Botryosphaeriales: Botryosphaeriaceae), a common gall endophyte recorded in France, Canada, USA, South Africa, Australia and Japan (Bissett & Borkent 1988; Bronner 1992; Rohfritsch 1992, 2008; Adair *et al.* 2009; Kobune *et al.* 2012). In addition to *B. dothidea*, four other fungal genera isolated from galls, *Epicoccum, Alternaria, Phoma* and *Fusarium*, have been reported (Adair *et al.* 2009).

Besides the five *Daphnephila* in Taiwan, there are three named species in India and one in Japan (Kieffer 1905;

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Yukawa 1974). Many undescribed species were found in Thailand, Indonesia, and Japan (Tokuda & Yukawa 2007; Tokuda *et al*. 2008) and *Daphnephila* may therefore be widely distributed in the eastern Palearctic and Oriental regions with species on *Machilus*, including *Persea*, *sensu* Chanderbali et al. (2001) and Li *et al.* (2011). The *Daphnephila–Machilus* association could be considered an impressive example of species diversification on a single host-plant taxon, similar to that noted for other genera of the Cecidomyiidae (e.g., Skuhravá 1986; Roskam 1992; Yukawa *et al*. 2005; Gagné 2008; Tokuda 2012; Gagné & Moser 2013; Gagné & Jaschhof 2014).

Machilus is a difficult group for determination of species, due to the morphological similarities among congeners, and it has been suggested that gall morphology of *Daphnephila* species may provide clues for *Machilus* determination (Yang *et al*. 2002). *Machilus zuihoensis* Hayata and *M. mushaensis* Lu are closely related species, endemic to Taiwan, and are geographically isolated from each other by the isothermal line of an average annual temperature of 18.5±0.5°C (Lu & Chen 1996). *Machilus zuihoensis* grows at warmer altitudes, usually below 600, 800, and 1100 m asl. in Northern, Central, and Southern Taiwan, respectively, whereas *M. mushaensis* occurs at cooler altitudes, usually higher than 1100 m elevation. However, the two species of *Machilus* may co-exist around the isothermal line. We have observed urn-shaped galls that occur abundantly on leaves of *M. zuihoensis* (Fig. 1A) and occasionally on *M. mushaensis* (Fig. 1B), but never on *M. thunbergii.* Until now the taxonomy of this gall-inducing cecidomyiid has remained unclear.

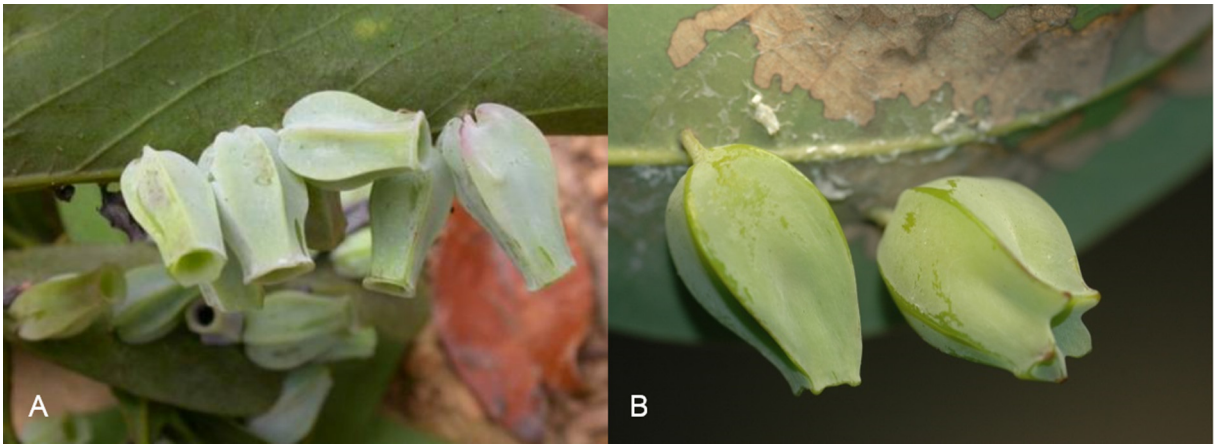

FIGURE 1. Similar urn-shaped galls (mature phase) induced by *D. urnicola* **sp.nov.** on the leaves of two different species of *Machilus* (Lauraceae). **A**. *M. zuihoensis*. **B**. *M. mushaensis*.

The purposes of this paper are to describe the new species of *Daphnephila* that induces urn-shaped galls on the leaves of *M. zuihoensis* and *M. mushaensis,* based on morphological and molecular evidence; to characterize the life-history of this species; to provide details on gall development, associated fungi, and parasitoids; and to compare the above details between populations of the taxa from galls on the leaves of *M. zuihoensis* and *M. mushaensis*.

Material and methods

Collection, life history observation, and preservation of galls and insects. Twigs of *M. zuihoensis* and *M. mushaensis* bearing leaves with galls were collected from 53 sites all over Taiwan between 2002 and 2011 (Fig. 2; Appendix 1).

Life-history information of *Daphnephila* from *M. zuihoensis* and *M. mushaensis* populations were recorded through field observation at four sites (Sun Moon Lake, Gukeng, Lien-Hua-Chih, and Dasyueshan) in Central Taiwan from 2002 to 2003, and at 53 sites (Appendix 1) from 2007 to 2011. Galls collected in different seasons of 2002–2003 and 2007–2011 were measured (Fig. 3) and dissected to examine the developmental stages of the two host populations. From 2007–2011 twigs bearing mature galls were inserted in vials with water, and placed in acrylic boxes (27 x 18 x 5 cm^3), to rear adults and their parasitoids under laboratory conditions. After emergence, 238 adults were placed individually in 4 ml expansion glass shell vials (Kimble 60965D-1) covered with punctured Parafilm® (Pechiney PM-996), and kept at room temperature (25±3°C) and relative humidity (50±5%) to observe adult life span.

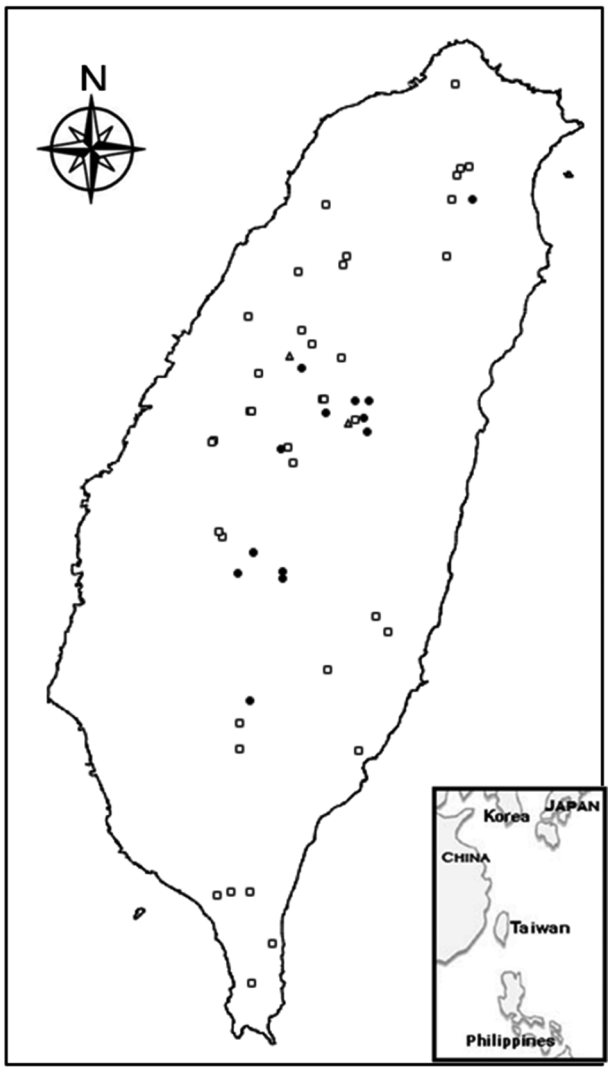

FIGURE 2. Map of Taiwan showing collecting sites of urn-shaped galls induced by *Daphnephila urnicola* **sp.nov.** on *M. zuihoensis* and on *M. mushaensis*. □ site with only *M. zuihoensis* population, ● site with both *M. zuihoensis* and *M. mushaensis* populations, △ site with only *M. mushaensis* population.

Adult behavior information for the two host populations was documented through field observation in the spring time (March to May) of 2009–2010 in Northern Taiwan (Wulai, and Pao-Shan Reservoir) and in Central Taiwan (Sihu, Huisun Forest Recreation Area, and DaKeng Hiking Trails).

Adults were preserved in 70% ethanol for morphological study and in 99.5% ethanol for molecular analysis. Parasitized gall cecidomyiid pupae were also obtained from dissected galls and were diagnosed as endo- or ecto-parasitoids from multiple observations. Adult parasitoids were preserved in 70% ethanol and determined by Kazunori Matsuo (Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Japan).

Slide preparation. Slide-mounted specimens were prepared for morphological examination following Gagné (1994). They were examined and illustrated under a light microscope (Olympus BX 50, Japan) fitted with a drawing tube. Photos were taken using AutoMontage with a compound microscope (Leica DMR, Germany). Morphological terminology follows McAlpine (1981), Gagné (1994), Tokuda (2004) and Tokuda *et al.* (2004, 2008) for the adults and pupae, and Möhn (1955), Yukawa (1971), and Mamaev & Krivosheina (1993) for the larvae. The holotype and 37 paratypes of the newly described species are deposited at the Department of Entomology, National Chung Hsing University, Taiwan (NCHU). Two paratypes are deposited at Kyushu University (ELKU), Japan, and two at the National Museum of Natural Science (NMNS), Taiwan.

Molecular analysis. DNA was extracted from the whole larval body or two legs of adult with 50 µl of Solution 1.0 in QuickExtract Tissue Kit (Epicentre Biotechnologies, Madison, WI) following the manufacturer's

instructions. A partial region of the cytochrome oxidase subunit I (COI) gene of mitochondrial DNA was amplified by polymerase chain reaction (PCR). Primers designed in this study are based on the mtDNA information of *Simosyrphus grandicornis* (Diptera: Syrphidae) and some dipteran COI sequences (Cameron *et al*. 2007). The forward is Diptera-49F (5'-AATCATAAAGATATTGGAAC-3') and the reverse is Diptera-734R (5'-CAACATTTATTTTGATTTTTTGG-3') which amplified a 643 bp fragment. The amplifications were done in 2 µl dNTPs mix (2.5 mM), 2.5 µl Taq polymerase reaction buffer (10X buffer), 0.5 µl Prime Taq , 0.5µl of each primer (10 µM), 18 µl double distilled water and 1 µl sample DNA making a final volume of 25 µl. The mixtures were incubated for 2 min at 94°C and then for 35 cycles consisting of 94°C for 45 sec, 45°C for 50 sec and 72°C for 50 sec. The final extension was at 72°C for 10 min and then held at 4°C. Electrophoresis was performed using 1% agarose gel. The purification of amplification products was carried out by QIA quick Gel Extraction Kit (Qiagen, Hilden, German) for direct sequencing.

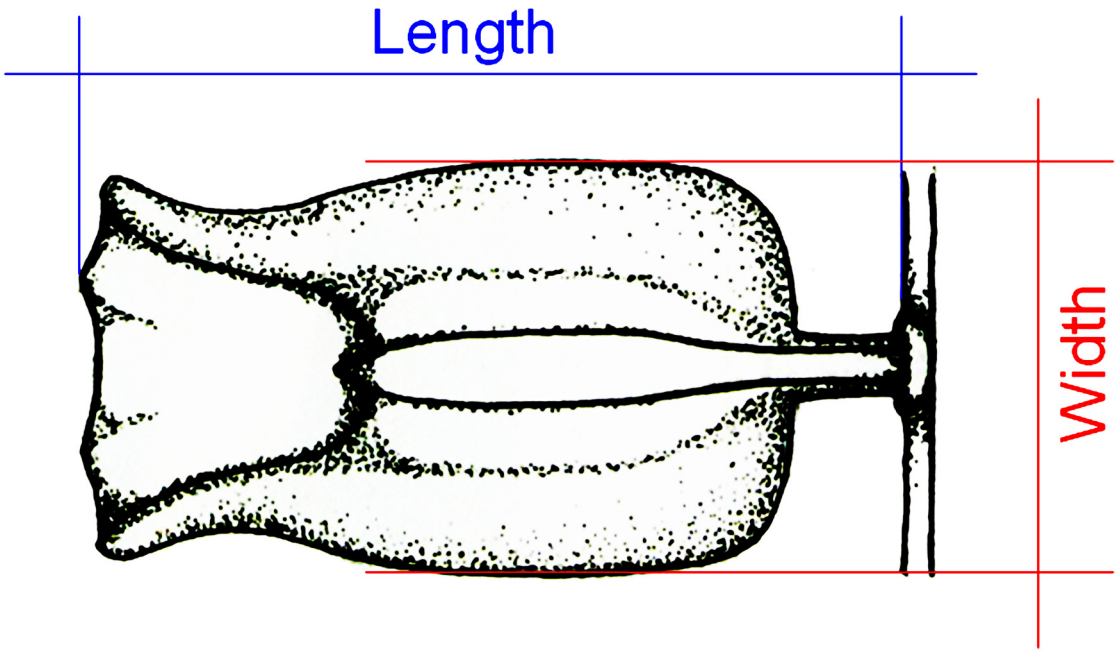

FIGURE 3. Reference points for gall measurements.

Nucleotide sequences were deposited in DNA Data Bank of Japan (DDBJ) with accession numbers of AB857360–AB857367. Sequences of three taxa from the same tribe downloaded from DDBJ were used as outgroups. Two of them, i.e., *Pseudasphondylia neolitseae* Yukawa (AB334237) and *Bruggmanniella actinodaphnes* Tokuda & Yukawa (AB334238), belong to Asphondyliina, and the other is *Oxycephalomyia styraci* (Shinji) (AB213402) from the Schizomyiina, the nearest clade to the Asphondyliina (Tokuda & Yukawa 2007). Moreover, the sequences of five *Daphnephila* species from Taiwan (AB334222-AB334228) and *D. machilicola* Yukawa from Japan (AB334217- AB334221) were also included in the ingroup.

DNA sequences were aligned using ClustalW (Thompson *et al*. 1994) implemented in BioEdit (Hall, 1999). Phylogenetic analysis was performed by the neighbor-joining (NJ) clustering method using MEGA 6 (Tamura *et al*. 2013). Pairwise deletion in proportional distance and Kimura two-parameter divergence were used in neighbor-joining inference (Kimura 1980). The most parsimonious trees (MP) were determined under the heuristic search strategy, tree-bisection reconnection (TBR), and parsimous ratchet algorithm for 1,000 bootstrap resamplings by TNT v 1.1 (Goloboff *et al*. 2008).

Histology of gall tissue and fungal culture. Gall samples for histology were fixed in FAA solution (formalin: acetic acid: 90 % alcohol: water= 1: 1: 9: 9) and vacuumed for 1 hour ensuring a complete fixation of tissues. Samples were then softened in 4% ethylenediamine for 1–3 days (modified from Carlquist 1982), and further dehydrated through a series of *tert*-Butyl alcohol–ethanol, followed by paraffin infiltration and embedding. Embedded samples were sectioned at 9 µm thickness. Sections were stained in 1% safranin O – 0.5% fast green following the routine protocol (*sensu* Johansen 1940; Hsiao *et al.* 1995).

Fungal mycelia isolated from the larval chambers of dissected galls were cultured in sterile Petri dishes with potato dextrose agar at 25°C. The potato dextrose agar was prepared by heating a mixture of 39 g potato dextrose broth, 5.1 g agar, and 1,000 ml distilled water, and autoclaving for at least 40 min. The solution was then poured into the Petri dishes, and kept at room temperature (25°C) for 2–3 days for solidification. Cultured mycelia were harvested after 7–10 days and identified by Chi-Yu Chen (Department of Plant Pathology, National Chung Hsing University, Taiwan).

Results

Taxonomy

Based on morphological comparison, molecular analysis, and biological data, it is clear that the gall midge that induces the urn-shaped gall on leaves of *M. zuihoensis* and *M. mushaensis* in Taiwan is a new species of *Daphnephila*. The new species is described below and the related biological information is provided in the later sections.

Daphnephila urnicola Chiang, Yang & Tokuda sp. nov.

MALE (Fig. 4B, D–G) Eye bridge 3–4 facets long medially. Among the twelve flagellomeres, first flagellomere 175–190 μm long, 3.1–3.5 times as long as wide, 1.1–1.2 times as long as second; fifth flagellomere 160–175 μm long, 3.2–3.5 times as long as wide; twelfth flagellomere 140–145 μm long, 2.8–4.3 times as long as wide. Frontoclypeus with 26–27 setae. Labella hemispherical in lateral view, with 32–37 setae. Palpus three-segmented, first segment globose in ventral view, about 29 μm long; second about 1.5 times as long as first; third about 1.2 times as long as second; distal two palpal segments sometimes more or less fused.

Wing length about 3.0 mm long, 2.3–2.4 times as long as wide. Scutum with two rows of dorsocentral setae; anteriorly with 38–39 dorsolateral setae, posteriorly with 44–50 dorsolateral setae. Anepisternum with 15–17 scales, mesepimeron with 44–75 setae.

First through seventh abdominal tergites rectangular, eighth tergite not sclerotized. Second through seventh abdominal sternites 0.42–0.50 times as long as wide; eighth sternite small, 0.79 times as wide as seventh sternite, about 0.60 times as long as wide.

Terminalia: hypoproct incised by shallow or deep emargination, forming a pair of lobes, each lobe with a few apical setae; mediobasal lobe shorter than cerci; aedeagus nearly parallel sided, truncate apically.

FEMALE (Fig. 4A, C). Frontoclypeus with 17–23 setae. First flagellomere 120–200 μm long, 2.4–3.6 times as long as wide, about 1.1–1.2 times as long as second; fifth flagellomere 133–145 μm in length, 2.9–3.2 times as long as wide; distal flagellomeres shortened and terminal one subglobular.

Wing length about 2.3 mm, 2.3–2.5 times as long as wide. Anterior dorsolaterally with 35–51 setae, posterior dorsolaterally with 48–58 setae. Anepisternum with 17–20 scales, mesepimeron with 43–60 setae.

First through seventh abdominal tergites and second through sixth sternites as in male. Seventh abdominal sternite setose, 520–560 μm long, about 1.1–1.3 times as long as wide.

Ovipositor short, with many long setae ventrally on apical half; cerci-like structure bilobed, situated on anterior of intersegmental membrane between eighth and ninth abdominal segments.

MATURE LARVA (Fig. 4H). Body light yellow. Second antennal segment short, subglobular; two cervical papillae each with a minute seta. Spiracle present on prothorax and first through seventh abdominal segments, invisible on eighth abdominal segment; four dorsal papillae present on thoracic segments, each with a minute seta; two dorsal papillae present on first through seventh abdominal segments, each with a minute seta; terminal papillae not apparent. Sternal spatula semicircular, anteriorly with two slender, acutely pointed lobes, each lobe 60–70 μm long; two lateral papillae present on thoracic segments, each with minute seta. First through seventh abdominal segments with two posterior ventral papillae, each with a minute seta; terminal papillae not apparent.

PUPA (Fig. 4I). Body length 3.1–4.1 mm. Length from the base of antennal sheath to the tip of apical horn 295–320 μm long; prothoracic spiracle 145–230 μm long; abdominal spiracles on second to seventh segments

about 22–35 μm long; second through eighth abdominal segments with 5–7 transverse rows of rather long spines on anterior half of dorsal surface, each spine 16–38 μm long.

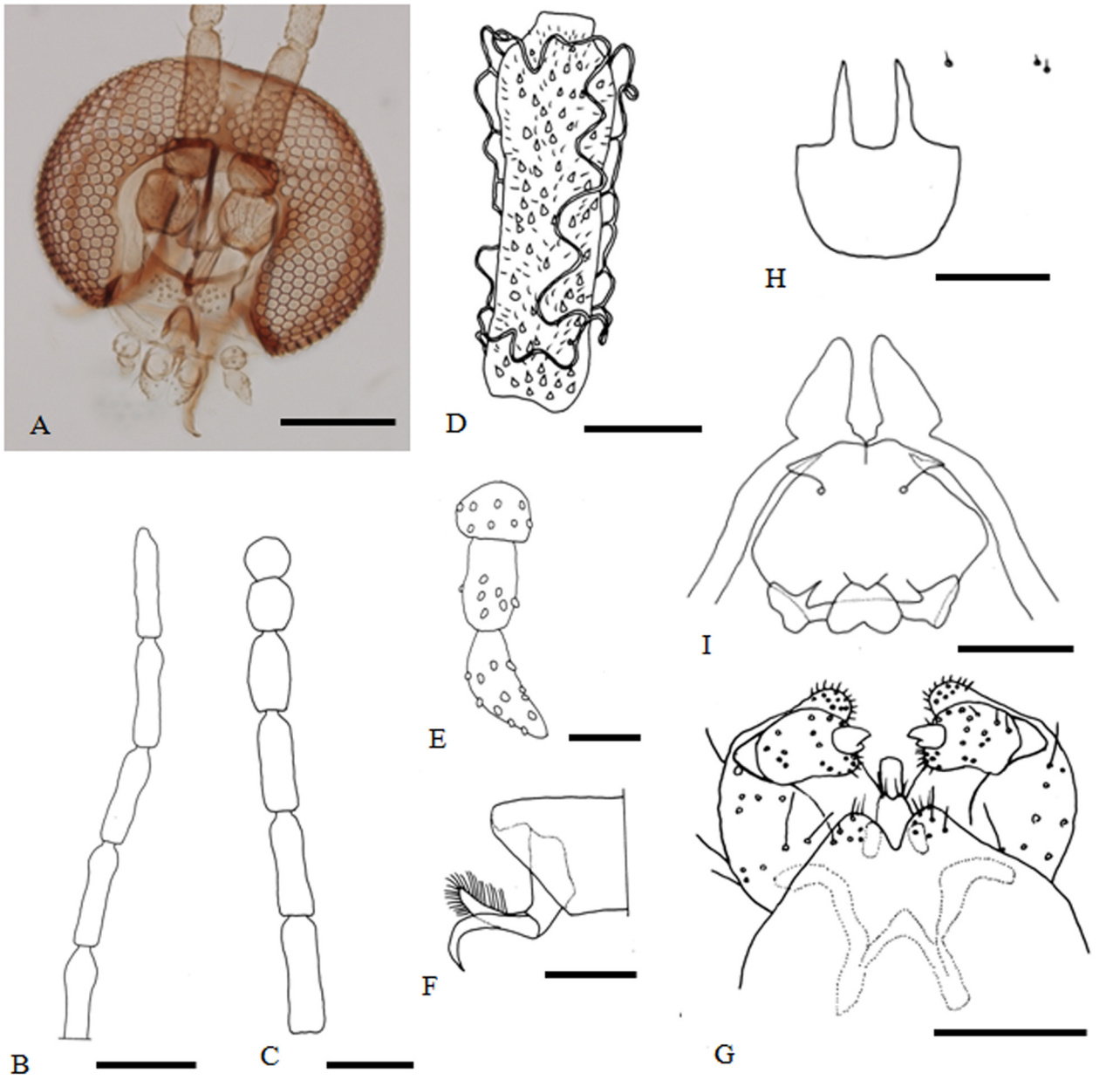

FIGURE 4. *Daphnephila urnicola* **sp. nov. A**. female head, frontal view, scale bar= 0.6mm. **B**. male flagellomeres 8–12, scale bar= 0.25mm. **C**. female flagellomeres 7–12, scale bar= 0.25mm. **D**. male flagellomere 5, scale bar= 0.05mm. **E**. male palpus, scale bar= 0.05mm. **F**. male hind tarsal claw and empodium, scale bar= 0.04mm. **G**. male terminalia, dorsal view, scale bar= 0.1mm. **H**. larva, sternal spatula and adjacent papillae, ventral view, scale bar= 0.05mm. **I**. pupa, head, frontal view, scale bar= 0.25mm.

Material examined. *Holotype*: ♂ (on slide, NCHU2013004-001, deposited in NCHU) emerged on 18 March 2008 from a gall on *M. zuihoensis* collected by T. C. Chiang on 17 March 2008 from Lung-Ding Ancient Road (323m asl.), Changhua, Taiwan.

Paratypes: 8 ♂ & 1 ♀ (on slides, NCHU2013004-002 to 010; NCHU2013004-002 (1♂) and 010 (1♀) deposited in NMNS and others deposited in NCHU), same data as holotype; 4 ♀ (on slides, NCHU2013004-011 to 014, deposited in NCHU), Lung-Ding Ancient Road (374m asl.), Changhua, Taiwan, same data as holotype; 2 mature larvae (on slides, NCHU2013004-015 to 016, deposited in NCHU), Lung-Ding Ancient Road (323m asl.), Changhua, Taiwan, same data as holotype; 2 mature larvae (on slides, NCHU2013004-017 to 18, deposited in

NCHU), collected by Y.T. Hong on 15 January 2008 from same locality as holotype; 2 ♂ & 2 ♀ (on slides, NCHU2013004-019 to 022, NCHU2013004-019 (1♂) deposited in ELKU and others deposited in NCHU), collected by T. C. Chiang on 16 March 2008 from Chushui Lane (349m asl.), Changhua, Taiwan; 3 ♂(wet in 70% Ethanol, NCHU2013004-023 to 025; deposited in NCHU), 2 ♀ (wet in 70% Ethanol, NCHU2013004-026 to 027; deposited in NCHU), 1 ♂ (on a slide, NCHU2013004-028; deposited in NCHU) & 1 ♀ (on a slide, NCHU2013004-029; deposited in NCHU) from galls on *M. zuihoensis* collected by T. C. Chiang on 16 March 2008 from Sihu (395m asl.), Miaoli, Taiwan; 2 ♀ (on slides, NCHU2013004-030 to 031; deposited in ELKU and NCHU), from galls on *M. zuihoensis* collected by T. C. Chiang & W. N. Chen on 26 March 2009 from Da-Han woodland path (640m asl.), Pingtung, Taiwan; 1 mature larva & 2 pupae (on slides, NCHU2013004-032 to 034; deposited in NCHU) from galls on *M. zuihoensis* collected by T. C. Chiang & W. N. Chen on 26 March 2009 from Lilishan (882m asl.), Pingtung, Taiwan; 1 ♀ & 1 pupa (on slides, NCHU2013004-035 to 036; deposited in NCHU) from galls on *M. zuihoensis* collected by T. C. Chiang on 20 April 2008 from Lilishan (873m asl.), Pingtung, Taiwan; 1 mature larva (on a slide, NCHU2013004-037; deposited in NCHU) from a gall on *M. zuihoensis* collected by T. C. Chiang on 16 February 2010 from Pi-Lu Zen Temple (295m asl.), Houli, Taiwan; 1 ♂ & 1 mature larva (on slides, NCHU2013004-038 to 039; deposited in NCHU) from galls on *M. mushaensis* collected by T. C. Chiang on 19 March 2008 from Dasyueshan (1393m asl.), Taichung, Taiwan; 1 ♂ & 1 mature larva (on slides, NCHU2013004-040 to 041; deposited in NCHU) from galls on *M. mushaensis* collected by T. C. Chiang on 19 March 2008 from Dasyueshan (1418m asl.), Taichung, Taiwan; 1 pupa (on a slide, NCHU2013004-042; deposited in NCHU) from a gall on *M. mushaensis* collected by T. C. Chiang on 02 March 2010 from Gao-Feng Lane (1234m asl.), Nantou, Taiwan.

Distribution. [Taiwan] 300–1300 asl. primary and secondary forest (Fig. 2).

Host plants. *Machilus zuihonensis* Hayata and *M. mushaensis* Lu (Lauraceae).

Galls. Urn-shaped, with 3–7 vertical ridges along the outer surface, hypophyllous (Fig. 1), remain green from young to dehiscent stages, single chambered.

Etymology. The specific name, *urnicola,* refers to the urn-shaped (*urna*) gall that the species induces and dwells (*cola*) in.

Remarks. In terms of morphology, the new species can be distinguished from the other *Daphnephila* as follows. The Indian *D. haasi* Kieffer, *D. glandifex* Kieffer and *D. linderae* Kieffer were described as having the posterior margin of the hypoproct entire (Yukawa 1974), whereas in *D. urnicola* the hypoproct has a shallow incision. The new species is distinguishable from five Taiwanese *Daphnephila* associated with *M. thunbergii* by the following characters: from *D. ornithocephala* Tokuda, Yang & Yukawa by the four-segmented palpus; from *D. stenocalia* Tokuda, Yang & Yukawa by the acute pupal antennal horns; from *D. sueyenae* Tokuda, Yang & Yukawa by the longer mediobasal lobe; from *D. taiwanensis* Tokuda, Yang & Yukawa by the presence of setose pupal apical papillae and longer female seventh abdominal sternite; from *D. truncicola* Tokuda, Yang & Yukawa by the shape of the larval sternal spatula, setose pupal apical papillae, and wider aedeagus. *Daphnephila machilicola* from Japan differs from *D. urnicola* in having pupal lower facial papillae (Yukawa 1974). In addition, it should be noted that unlike other species, spiracles of the larval eighth abdominal segment are not apparent in *D. urnicola*. This character may be useful for future taxonomic studies of this genus.

TABLE 1. The minimum and maximum pairwise distances within *D. urnicola* and between *D. urnicola* and other *Daphnephila* species.

pairwise distances	minimum	maximum
D. urnicola	0.00%	0.47%
D. stenocalia	0.70%	1.24%
D. machilicola	1.64%	2.50%
D. ornithocephala	2.61%	3.02%
D. sueyenae	2.83%	3.51%
D. taiwanensis	6.03%	6.65%
D. truncicola	11.21%	11.90%

Both the NJ and the MP trees showed a similar topology with higher resolution in the NJ tree. Here we present the most parsimonious inference, the consensus from 3 trees of length of 223, with the bootstrapping values of both NJ and MP clustering method (Fig. 5). The taxa of the *M. zuihonensis* and *M. mushaensis* populations formed a monophyletic group and were supported by relatively high bootstrap values (83%, Fig. 5). The minimum and maximum pairwise distances were 0 and 0.47%, respectively inside the *D. urnicola* group (Table 1). The sister taxon of *D. urnicola* populations from *M. zuihoensis* and *M. mushaensis* is *D. stenocalia*, which forms longitudinal club-shaped galls on *M. thunbergii* in NJ tree.

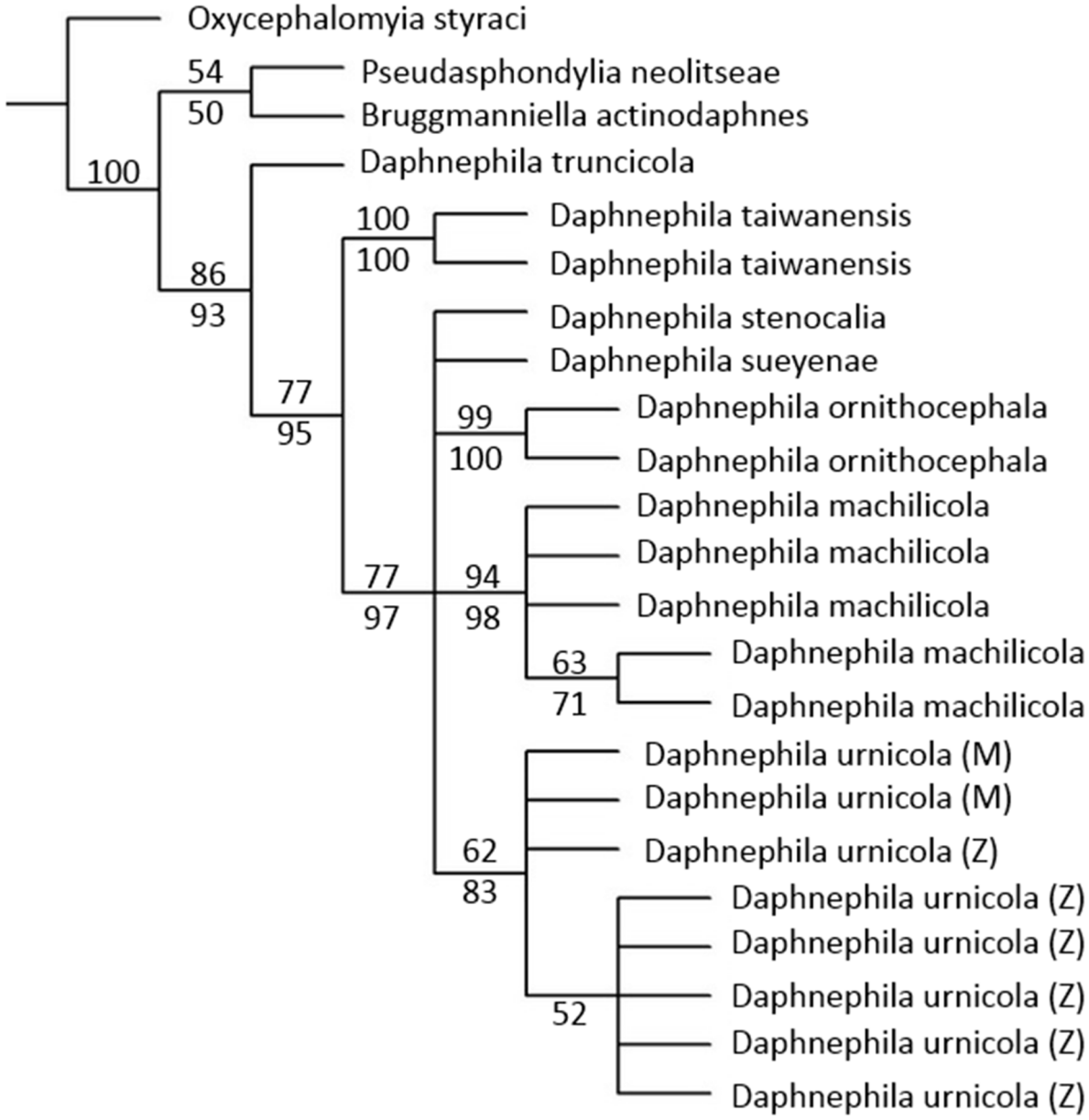

FIGURE 5. Strict consensus tree of *Daphnephila* species relationships based on COI gene data. Three equally parsimonious trees were acquired (length: 223). Bootstrap values greater than 50% for the MP inference, and the neighbor-joining (NJ) method, are shown above and below the branch, respectively. Individual specimens of *D. urnicola* collected from *Machilus mushaensis* and *M. zuihoensis* are labeled with M and Z in parentheses, respectively.

Biology. *Life cycle and development of gall tissues*. Both *M. zuihonensis* and *M. mushaensis* populations of *D. urnicola* were univoltine, inducing single-chambered galls along the abaxial surfaces of the lamina, and completing their annual life cycle on either *M. zuihoensis* or *M. mushaensis*. These urn-shaped galls have never been found on other *Machilus* spp. in Taiwan, such as *M. thunbergii* and *M. kusanoi,* which usually coexist with *M. zuihoensis* or *M. mushaensis* in broad-leaved evergreen forests.

In field situations, mating usually occurred in the morning, and oviposition occurred between 1500 and 1700 h. Females walked along primary veins of freshly expanding leaves of *M. zuihoensis* and *M. mushaensis* and laid eggs in inter-vein locations between primary and secondary veins on the under surface. Occasionally oviposition occurred along the under surface of the tip of newly opened leaf buds. In 4–7 days, the neonate first-instar larvae penetrated into the leaf tissue, where they entered summer diapause (Fig. 6A). There was no active proliferation of cells, but hyphae could be found inside the gall chamber at this stage (Fig. 6B).

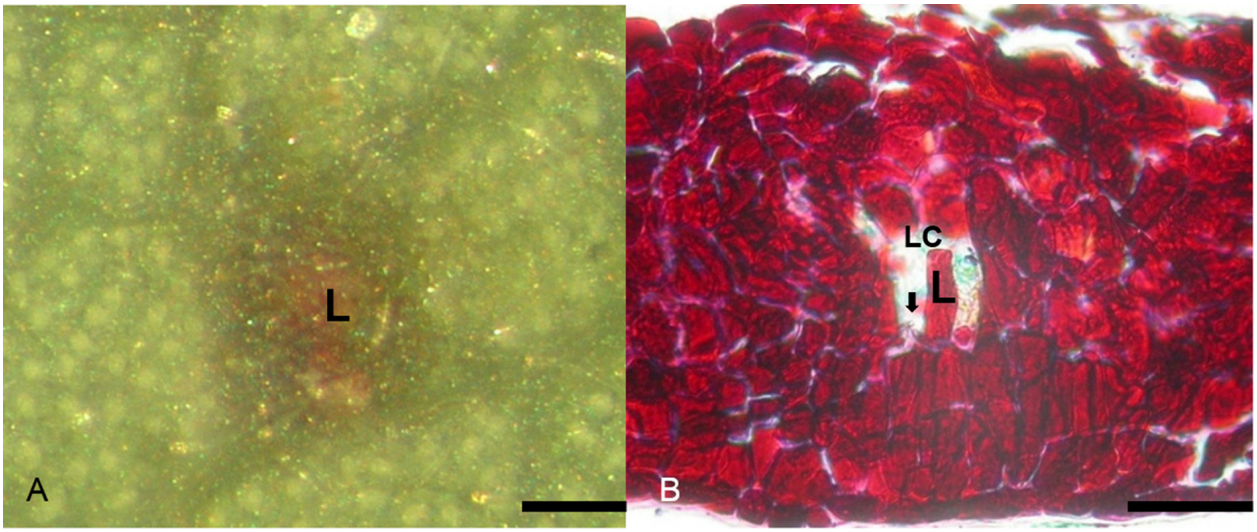

FIGURE 6. Diapausing first instar larva of *Daphnephila urnicola* **sp.nov.** in the leaf tissue of undeveloped galls on *Machilus zuihoensis*. **A**. the appearance of the adaxial surface of the leaf with first instar larva inside. **B**. cross section of leaf tissues showing initial gall with larval chamber and fungi. L= Larva, LC= larval chamber, and arrow= hyphae.

The developmental processes of galls induced by *D. urnicola* on *M. zuihonensis* and *M. mushaensis* are similar. Galls started to develop and protruded from the abaxial side of leaves in autumn. They developed rapidly and broke through leaf tissue when the larvae molted into the second instars (Fig. 7A & B). Gall tissue was about 15 cell-layers in width and could be differentiated into (1) epidermis, (2) cortex, (3) vascular traces, and (4) nutritive tissue including the parenchyma layer and fungus layer within (Fig. 8). The parenchyma cells in the cortex were filled with tannin and some secretory cells existed among them. The galls grew along the vertical axis, transforming the spherical projection beyond the leaf surface to grow like a column (Fig. 7C & D). The second instar larva remained at the basal part of the gall.

Most *D. urnicola* reached third instars in the galls during winter (Fig. 7E & F). Exceptionally a few individuals from the southern populations remained as second instar larvae and a few from the northern populations reached the pupal stage. Galls were 40–50 cell-layers in width, with an organization similar to that during the occupation of the second instar larva, except for the differentiation of sclerenchyma between vascular bundles and the nutritive tissue (Fig. 9). At the end of the growth and differentiation phase, around January, cells at the base of galls grew along the vertical axis of the gall, and those at the top of galls grew along the lateral axis. The differentiated gall tissues were heterogeneous at the basal part of the gall, sclerenchyma occurred between vascular bundles and the nutritive tissue, whereas at the terminals, only parenchyma cells and a few secretory cells occurred.

In late winter (January) and early spring (February), as the third instars pupated within galls (Fig. 1), sclerenchyma cells filled nearly half of the basal part of galls and the parenchyma layer inside the sclerenchyma layer was undetectable. Meanwhile, the shape of the operculum (the apical part of the gall), which opens at the time of emergence, was acute in angle on *M. mushaensis* (Fig. 10D) compared to those on *M. zuihonensis* (Fig. 10C). The number of parenchyma cell layers in this part of *D. urnicola* galls was 7–12 on *M. zuihonensis* (Fig. 10A) compared to 16–18 on *M. mushaensis* (Fig. 10B).

Adults emerged in March—April (spring) leaving the pupal exuviae at the opercular points of the gall (Fig. 10E & F). The gall tissue gradually dried and detached from the leaf. The life span of *D. urnicola* adults emerging in the laboratory and kept in vials was 4.13±1.66 (63 females) and 2.14±0.86 days (50 males) from *M. zuihonensis*; and 3.40±1.76 (53 females) and 2.49±0.73 days (72 males) from *M. mushaensis*. The longest life span observed was seven days for females from both hosts.

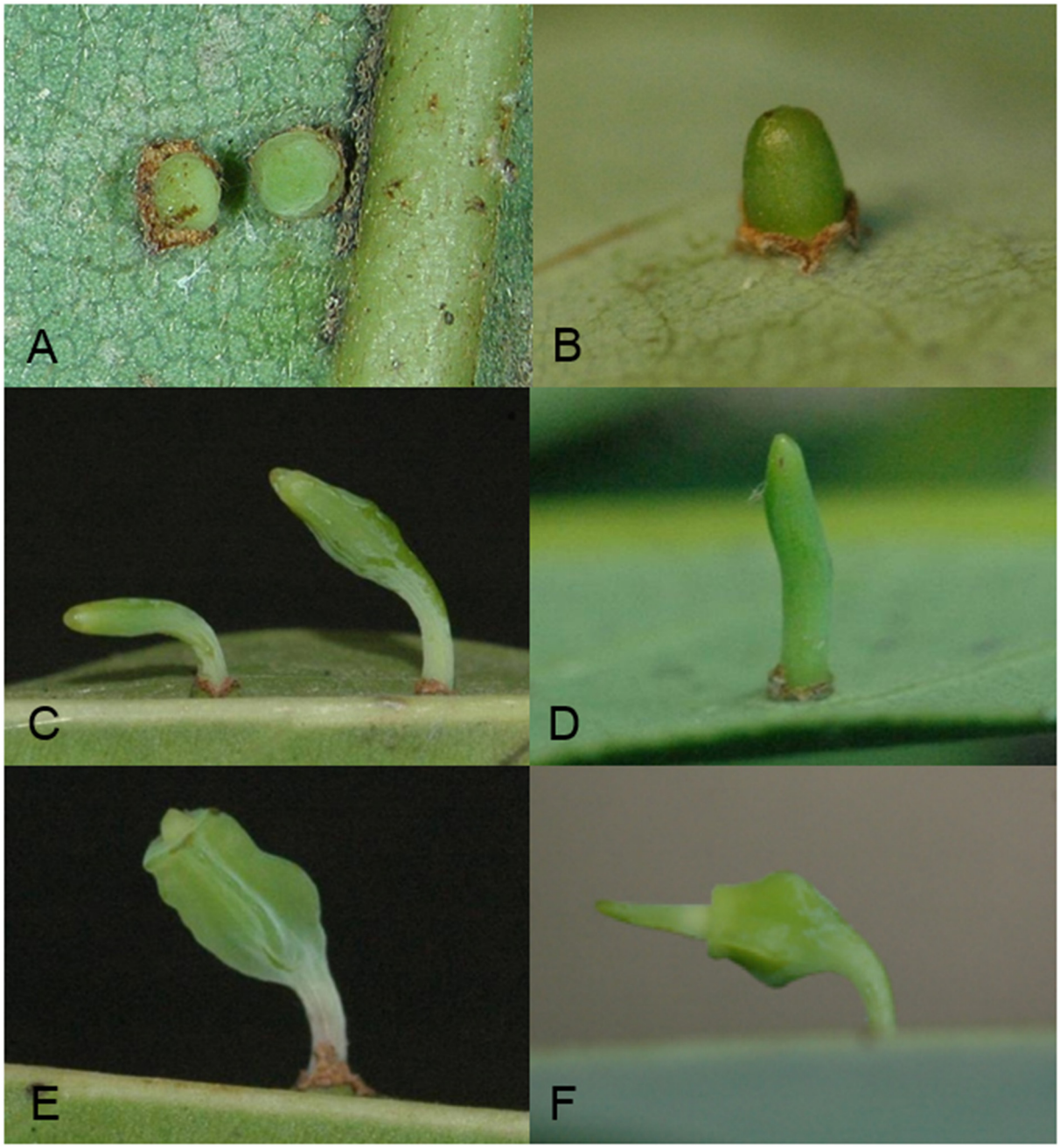

FIGURE 7. Development of urn-shaped galls on *Machilus zuihoensis* and *M. mushaensis* at various stages of growth and differentiation phase. **A**. early stage, on *Machilus zuihoensis*. **B**. early stage, on *M. mushaensis*. **C**. middle stage, on *M. zuihoensis*. **D**. middle stage, on *M. mushaensis*. **E**. later stage, on *M. zuihoensis*. **F**. later stage, on *M. mushaensis*. Arrows= suberized host tissue.

Measurements of galls. The *D. urnicola* galls on *M. mushaensis* were significantly larger than those on *M. zuihonensis* (ANOVA; F=14.57, p <0.001 for gall length and F=4.54, p <0.05 for gall width). The average length of galls on *M. zuihonensis* was 12.35 ± 2.76 mm, average width 6.61 ± 1.72 mm, and length–width ratio 1.87 (n=318). The average length of galls on *M. mushaensis* was 14.13 ± 1.90 mm, average width 7.23 ± 1.19 mm, and length–width ratio 1.95 (n=183).

Associated fungi. *Botryosphaeria dothidea* was isolated from almost all chambers of the *D. urnicola* galls induced on *M. zuihonensis* (98.68%) and *M. mushaensis* (97.37%). *Phomopsis* sp. (1.33%) was also isolated from galls on *M. zuihonensis*, and *Nigrospora* sp. (1.32%) and *Pestalotia* sp. (1.32%) from galls on *M. mushaensis*. The pouch on the females of *D. urnicola* is covered by an enlarged sheath on the 7th abdominal sternite (Fig. 11). However, under microscopic examination neither spores nor hyphae were found in the mycangia (pouch) of females just after emergence.

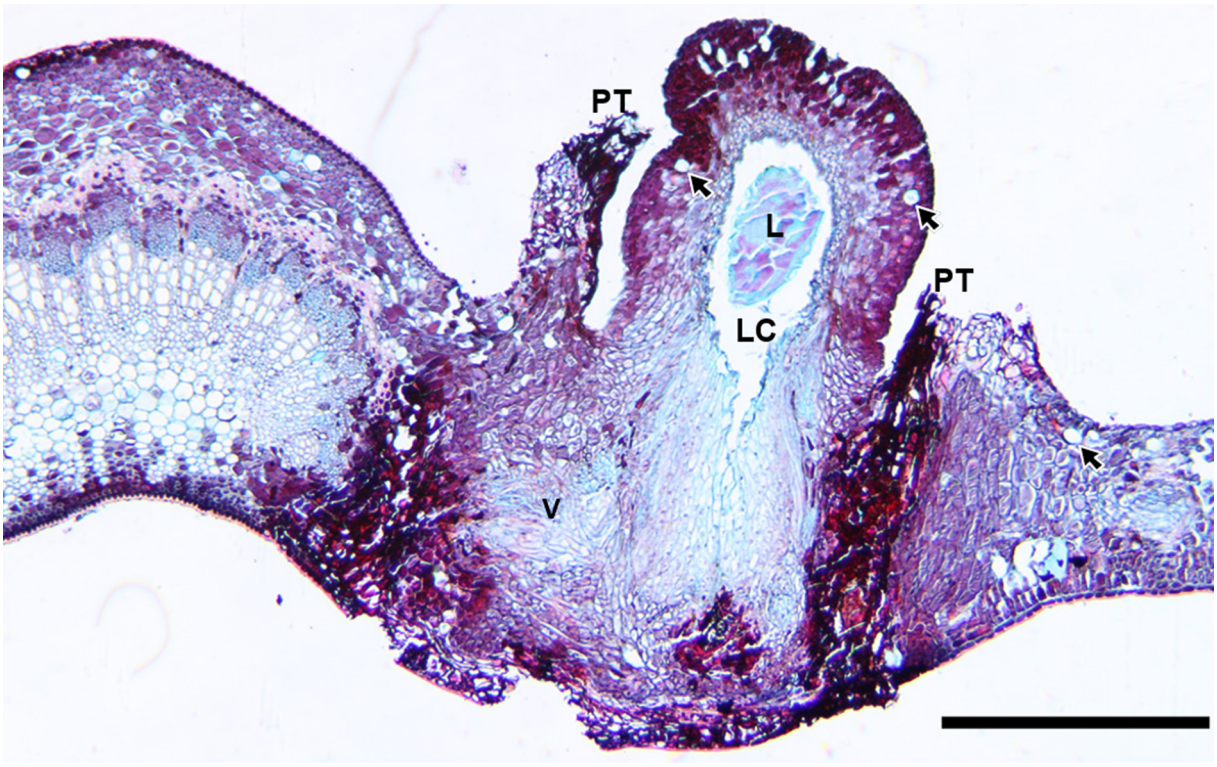

FIGURE 8. Tissue of urn-shaped galls on *Machilus zuihoensis* induced by *Daphnephila urnicola* **sp.nov.** at the middle stage of growth and differentiation phase (longitudinal section). Bar = 500 μm. L= Larva, LC= larval chamber, PT= plant tissue suberized, V= vascular tissue, arrow= secretory cells.

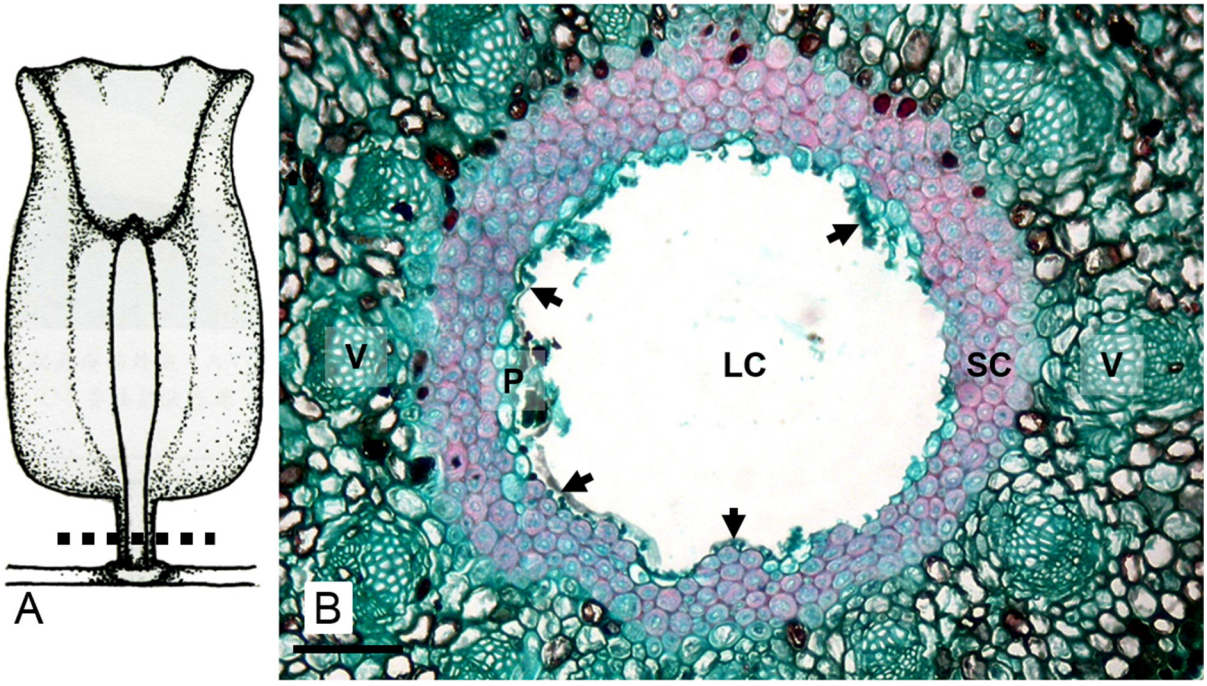

FIGURE 9. A. Dashed line showing plane of transverse section in B. **B**. Basal part of urn-shaped gall tissues on *Machilus zuihoensis* induced by *Daphnephila urnicola* **sp.nov.** at the later stage of the growth and differentiation phase (cross section). Bar = 50 μm. LC= larval chamber, SC= sclerenchyma cells, P= parenchyma layer, V= vascular bundles, arrow= hyphae.

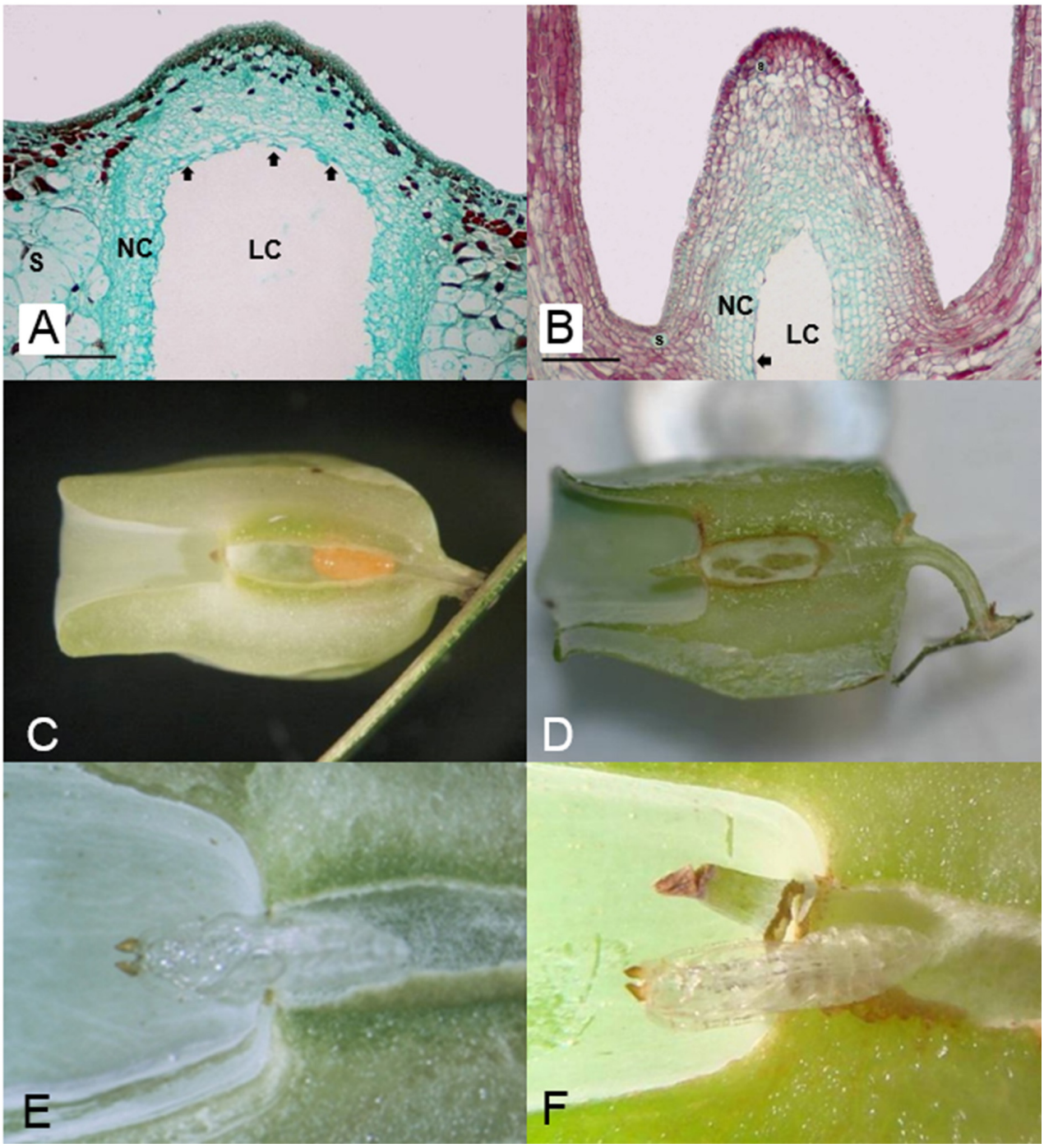

FIGURE 10. Pictures showing opening area of mature galls of *Daphnephila urnicola* **sp.nov. A**–**B**. Apex of gall (longitudinal section) on *Machilus zuihoensis* (A, Bar = 80μm) and on *M. mushaensis* (B, Bar = 180μm), LC= larval chamber, NC= nutritive cell, S= secretory cell.. **C**–**D**. Longitudinal section of entire gall on *Machilus zuihoensis* (C) and on *M. mushaensis* (D); **E**–**F.** emergence hole and pupa skin on *M. zuihoensis* gall (E) and on *M. mushaensis* gall (F).

Parasitoids. Three endoparasitoids, *Leptacis* sp., *Platygaster* sp. (both Hymenoptera: Platygastridae), and *Gastrancistrus* sp. (Hymenoptera: Pteromalidae), and six species of ectoparasitoids, *Bracon* sp., *Simplicibracon curticaudis* (both Hymenoptera: Braconidae), *Sigmophora* sp. (Hymenoptera: Eulophidae), *Eupelmus* sp. (Hymenoptera: Eupelmidae), *Ormyrus* sp. (Hymenoptera: Ormyridae), and a pteromalid species were obtained from *D. urnicola* galls on *M. zuihonensis*. In contrast, only *Platygaster* sp. and *Eupelmus* sp. were obtained from galls on *M. mushaensis*.

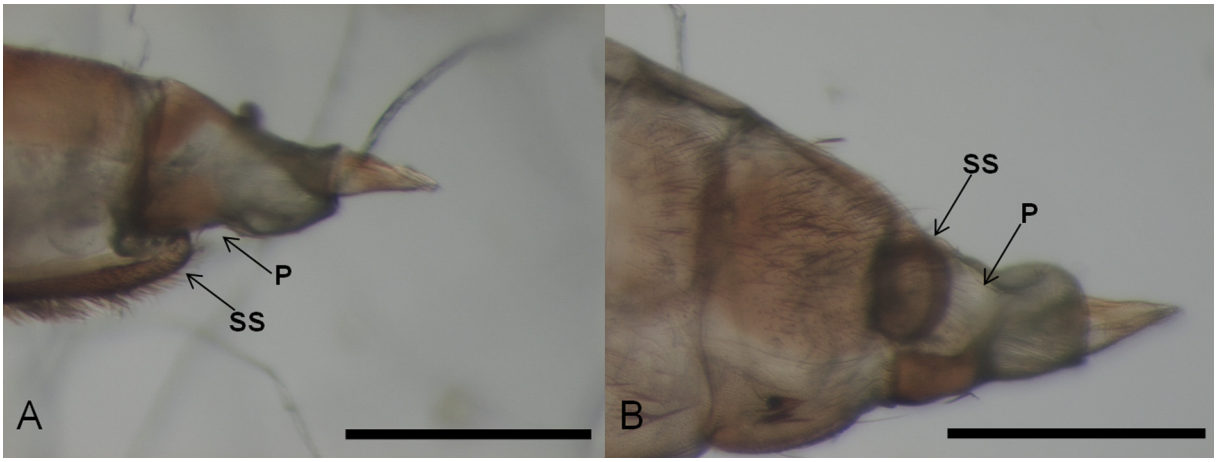

FIGURE 11. Female terminalia showing the pouch structure, a mycangium-like fungal spore-carrying organ, of *Daphnephila urnicola* **sp. nov.** The structure is covered by an enlarged sheath on the 7th abdominal sternite. **A**. Lateral view, scale bar= 0.5 mm. **B**. Ventral-lateral view, scale bar= 0.5 mm. P= pouch and SS= seventh sternite.

Discussion

Daphnephila urnicola **sp. nov.** is the sixth species of the *Daphnephila* gall midges described from Taiwan and the 10th species from the world. In addition, we have collected at least eight other morphospecies of *Daphnephila* that induce leaf, and stem galls on various *Machilus* species in Taiwan. Because other unnamed *Daphnephila* species seem to be associated with multiple *Machilus* hosts, and possibly induce polymorphic galls on them, further in depth examinations are needed to determine the actual number of species present.

Life-history strategies of univoltine gall midges (Diptera: Cecidomyiidae) have been recognized as of four types, in terms of their pupation sites and overwintering larval stages (Yukawa 1987; Yukawa & Rohfritsch 2005). In the type IIA life history strategy (*sensu* Yukawa 1987), the larvae develop less rapidly than those of species of the type IA or IB, but mature before winter. Full-grown larvae stay in galls that remain attached to the plant throughout winter and pupate within galls in the following spring. Species of *Daphnephila* are known to exhibit this strategy, as well as other species of *Ilex*-associated *Asteralobia*, *Euonymus*-associated *Masakimyia*, and *Pseudasphondylia,* which attacks various broad-leaved evergreen trees such as *Viburnum dilatatum* Thunb. (Caprifoliaceae), *Actinidia polygama* Sieb. et Zucc. (Actinidiaceae) and *Neolitsea sericea* (Bl.) Koidz. (Lauraceae) (Yukawa & Sunose 1976; Yukawa 1974; Tokuda *et al*. 2004; Tokuda *et al*. 2008; Tokuda 2012). *Daphnephila urnicola* also exhibits the type IIA life history strategy, overwintering as third instars or occasionally as pupae in cooler areas and as second instars in warmer areas. *Daphnephila machilicola* in Japan also has a type IIA life history strategy but overwinters as second instars (Maeda *et al*. 1982).

The developmental pattern and structure were mostly similar between *D. urnicola* galls on *M. zuihonensis* and *M. mushaensis*, except for the overall gall size and operculum shape. Both galls harbor the fungal species *B. dothidea* similar to other ambrosia galls. Weis (1982) showed that *B. dothidea* (syn. *Sclerotium asteris*) around gall chambers of *Asteromyia carbonifera* Felt (Cecidomyiidae) produced a stroma (tough, dense vegetative structure). However, we did not find similar stroma around *D. urnicola* larvae on *M. zuihoensis* and *M. mushaensis*. Our field observations indicated that the two thinner parts, basal stalk and terminal lid, of the galls were spots vulnerable to ectoparasitoid attacks. The sclerenchyma cell layers surrounding the basal part of the chamber may be able to protect larvae in the galls, which agrees with our observation that *D. urnicola* on *M. mushaensis*, with thicker tissues of the gall lid, was attacked by fewer ectoparasitoid species than those on *M. zuihoensis*. The exact function of the operculum structure in limiting parasitism deserves further examination.

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APPENDIX 1. Collection sites of *D. urnicola* **sp.nov.** in Taiwan.

County or City	Site	Latitude (N), Longitude (E)	Host plant
Taipei City	Yangmingshan National Park	121.524, 25.156	*M. zuihoensis*
	Tatongshan	121.563, 24.876	*M. zuihoensis*
New Taipei City	Shin-Shan Nursery	121.518, 24.849	*M. zuihoensis*
	Wulai	121.522, 24.826	*M. zuihoensis*
	Fushan Village	121.503, 24.780	*M. zuihoensis*
Hsinchu County	Pao-Shan Reservoir	121.032, 24.716	*M. zuihoensis*
	Taoshan elementary school	121.106, 24.577	*M. zuihoensis*
	Dalu Forest Road	121.098, 24.548	*M. zuihoensis*
Miaoli County	Shitan	120.931, 24.524	*M. zuihoensis*
	Sihu	120.752, 24.369	*M. zuihoensis*
Taichung City	DaKeng Hiking Trails	120.783, 24.178	*M. zuihoensis*
	Wushikeng	120.979, 24.279	*M. zuihoensis*
	Shalian Lane	121.097, 24.230	*M. zuihoensis*
	Tong-Lin	120.762, 24.049	*M. zuihoensis*
	Aoshan Trail	120.765, 24.047	*M. zuihoensis*
	Pi-Lu Zen Temple	120.739, 24.289	*M. zuihoensis*
	Motaining	120.948, 24.322	*M. zuihoensis*
	Tungmaoshan	120.953, 24.194	Both
	Dasyueshan	120.906, 24.237	*M. mushaensis*
Changhua County	Lung-Ding Ancient Road	120.628, 23.947	*M. zuihoensis*
	Chushui Lane	120.618, 23.944	*M. zuihoensis*
Nantou County	Bihu	121.149, 24.018	*M. zuihoensis*
	Sun Moon Lake	120.919, 23.871	*M. zuihoensis*
	Tangkungpei	121.024, 24.088	*M. zuihoensis*
	Lien-Hua-Chih Medicinal Herb Garden	120.898, 23.923	*M. zuihoensis*
	Chinglung Valley	121.032, 24.089	*M. zuihoensis*
	Mei-Feng	121.198, 24.083	Both
	Huisun Forest Recreation Area	121.037, 24.042	Both
	Chunyang	121.178, 24.021	Both
	Shenmu Forest Road	120.883, 23.506	Both
	Lien-Hua-Chih	120.875, 23.921	Both
	Nantzu Forest Road	120.879, 23.481	Both
	Aowanda National Forest Recreation Area	121.191, 23.975	Both
	Peitungyenshan	121.150, 24.083	Both
	Gao-Feng Lane	121.125, 24.006	*M. mushaensis*
Yunlin County	Gukeng	120.656, 23.620	*M. zuihoensis*
	County Road 194	120.648, 23.641	*M. zuihoensis*
Chiayi County	Shihmeng Valley	120.772, 23.571	Both
	Day Dong Mountain	120.716, 23.498	Both
Kaohsiung City	Tona Forest Road	120.726, 22.905	*M. zuihoensis*
	Fengkang log road	120.723, 22.993	*M. zuihoensis*
	Tengjhih National Forest Recreation Area	120.761, 23.068	Both

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APPENDIX 1. (Continued)

County or City	Site	Latitude (N), Longitude (E)	Host plant
Pingtung County	Shouchia Logging Road	120.842, 22.243	*M. zuihoensis*
	Lilishan	120.691, 22.421	*M. zuihoensis*
	Jinshueiying	120.764, 22.420	*M. zuihoensis*
	Da-Han woodland path	120.641, 22.405	*M. zuihoensis*
	County Road 199	120.771, 22.111	*M. zuihoensis*
Yilan County	Dulishan	121.486, 24.575	*M. zuihoensis*
	Fushan Botanical Garden	121.586, 24.764	Both
Hualien County	Walami Trail and Jiasin	121.225, 23.352	*M. zuihoensis*
	Nan'an	121.266, 23.301	*M. zuihoensis*
Taitung County	County Road 197	121.162, 22.896	*M. zuihoensis*
	Wulu	121.048, 23.173	*M. zuihoensis*