**Open Access** 

# Recent Divergence of *Neolitsea*-associated *Pseudasphondylia* Gall Midges (Diptera: Cecidomyiidae) with Description of a New Species from Taiwan

Sheng-Feng Lin<sup>1,₂,</sup>∗℗, Makoto Tokuda³℗, Man-Miao Yang²℗, Gene-Sheng Tung⁴℗, and Liang-Yu Pan⁴℗

<sup>1</sup>Department of Biology, National Museum of Natural Science, Taichung 404, Taiwan. \*Correspondence: E-mail: sflin654@gmail.com (Lin)
<sup>2</sup>Department of Entomology, National Chung Hsing University, Taichung 402, Taiwan. E-mail: mmy.letsgall@gmail.com (Yang)
<sup>3</sup>Department of Biological Resource Science, Faculty of Agriculture, Saga University, Japan. E-mail: tokudam@cc.saga-u.ac.jp (Tokuda)
<sup>4</sup>Forest Ecology Division, Taiwan Forestry Research Institute, Taipei 100, Taiwan. E-mail: gall@tfri.gov.tw (Tung); dowayoland@gmail.com (Pan)

urn:lsid:zoobank.org:pub:1716D744-2B26-4B49-89A5-B60391131593 Received 14 May 2024 / Accepted 14 February 2025 / Published 13 May 2025 Communicated by Y. Miles Zhang

A Taiwanese *Pseudasphondylia* species inducing spherical leaf galls on six *Neolitsea* hosts, viz, *N. acuminatissima*, *N. daibuensis*, *N. konishii*, *N. parvigemma*, *N. sericea* and *N. variabillima*, was regarded as a species allied to Japanese *P. neolitseae* Yukawa due to similarity of gall structure and host. The Taiwanese species is morphologically different from *P. neolitseae* in adult palpus segmental number, shapes of pupal antennal horn, pupal prothoracic spiracle, and larval sternal spatula, resulting in its description as a new species to science, *Pseudasphondylia hooki* sp. n. The species delimitation (Assemble Species by Automatic Partitioning method) supported that *P. hooki* sp. n. is distinct from *P. neolitseae*. The sistership of *P. hooki* sp. n. and *P. neolitseae* was also supported in the Maximum likelihood tree based on the 1<sup>st</sup> codon of *COI* region. The speciation event of them is assumed to be geographical isolation because the divergence corresponded to the separation of Taiwan and Japan in the Pleistocene. The patterns of ecological features (host species) on the phylogeny revealed that galling on *N. sericea* is primitive of *Neolitsea*-associated *Pseudasphondylia* lineage. Accordingly, the divergence pathway from north to south in Taiwan is suggested by the distributions of primitive (*N. sericea*) and most derived hosts (*N. daibuensis*).

Key words: Asphondyliina, Biogeography, East Asia, Phylogeny, Speciation

# BACKGROUND

Gall midges (Diptera: Cecidomyidiae) are the largest gall-inducing insect group and use host of various plant families worldwide, although some species are saprophagous, mycophagous or predaceous (Gagné and Jaschhof 2021). Some galling cecidomyiids display unique phenotypes in their gall characteristics, such as shape, galling part/position, and host plant spectrum, which are useful cues to explore the species identity comparing with known cecidomyiid congeners (Tokuda et al. 2004; Lin et al. 2020b 2022). Besides, evolution of these ecological features assists us to know the eco-speciation and biogeography of cecidomyiids, in particular to similar gall sorts occurring on related plants or in adjacent localities, as has been done in *Schizomyia* species (= *Asteralobia* syn. in Elsayed et al. 2018) on *Ilex* (Aquifoliaceae) (Tokuda et al. 2004), *Bruggmannilla* species on *Cinnamomum* (Lauraceae) (Lin et al. 2020b), and *Pseudasphondylia* species on

Citation: Lin SF, Tokuda M, Yang MM, Tung GS, Pan LY. 2025. Recent divergence of *Neolitsea*-associated *Pseudasphondylia* gall midges (Diptera: Cecidomyiidae) with description of a new species from Taiwan. Zool Stud **64**:09. doi:10.6620/ZS.2025.64-09.

Actinidia (Actinidiaceae) (Lin et al. 2022) in the East Asian Arc.

Pseudasphondylia neolitseae Yukawa, 1974 induces spherical leaf galls on Neolitsea sericea var. sericea (Lauraceae) and widely occurs in Japan (Honshu, Shikoku, Kyushu, and Southwest Islands) and southern Korea (Jeju Island) (Paik et al. 2004; Tokuda and Yukawa 2005). In Taiwan, undescribed Pseudasphondylia species are assumed to have an allied relationship with P. neolitseae due to the similar gall features and host affinity. Namely, the spherical leaf galls induced by unclarified Pseudasphondylia species have been noted from six Neolitsea species consisting of two native species, N. konishii and N. sericea var. sericea, and four endemic species, N. acuminatissima, N. daibuensis, N. parvigemma, and N. variabillima (Liang et al. 1999; Yang et al. 1999; Tung et al. 2018; Fig. 1). These provide an opportunity to explore the ecological divergence and island biogeography of *Neolitseae*associated *Pseudasphondylia* species.

In this article, the species identity of the Taiwanese *Pseudasphondylia* species on six *Neolitsea* host species was first clarified based on both morphological and molecular evidence. Then, we conducted phylogenetic analysis to address the following questions for *Neolitsea*-associated *Pseudasphondylia* species: (1) What is their possible divergence pathway in the East Asian Arc? (2) How did they diverse on *Neolitsea* plants? Clarifications of these two questions will contribute to the understanding of the divergence modes of cecidomyiids in this region.



Fig. 1. Leaf galls of *Pseudasphondylia hooki* sp. n. on *Neolitseae* species (A) *N. acuminatissima*, (B) *N. daibuensis*, (C) *N. konishii*, (D) *N. sericea* (E) *N. parvigemma*, and (F) *N. variabillima*.

## MATERIALS AND METHODS

## Collecting and preparation of specimens

Leaf galls induced by Neolitsea-associated Pseudasphondylia species were collected from various localities in Taiwan and Japan between 2009 to 2024. Larval, pupal, and adult specimens were mostly preserved in 70% ethanol after dissecting the galls or adult emergence. A part of the fresh specimens was kept in 95% ethanol for molecular studies. Slide-mounted specimens were prepared following Gagné (1994). Specimens were observed using an optical microscope (Leica<sup>®</sup> DM 750, Germany) and deposited in National Chung Hsing University, Taiwan (NCHU), National Museum of Natural Science, Taiwan (NMNS), and Taiwan Forestry Research Institute, Taiwan (TFRI). The morphological terminology of adult followed Gagné (2018) and Tokuda (2004), and that of larva and pupa followed Gagné (1994).

## Molecular analysis

Three individuals of P. neolitseae and 17 individuals of Taiwanese Neolitsea-associated Pseudasphondylia species were used in the present molecular work (Table 1). DNA extraction and PCR procedures followed Lin et al. (2019). The COI maker primers were referenced Cameron et al. (2007): Forward primer: Diptera-49F (5'-AATCATAAAGATATTGGAAC-3') and reverse primer: Diptera-734R (5'-CAACATTTATTTGATTT TTTGG-3'). The 20 COI sequences were successfully analyzed and deposited in the DNA Data Bank of Japan (DDBJ) with accession numbers shown in table 1. The COI sequences of Illiciomyia yukawai (LC348719), P. kiwiphila (LC710532), and P. rokuharensis (LC538357) were used as outgroups. The sequences were aligned using ClustalW (Thompson et al. 1994) with BioEdit (Hall 1999) and the species delimitation methods using ASAP (Assemble Species by Automatic Partitioning) online service (Puillandre et al. 2021) with Jukes-Cantor model. The genetic distances among *Pseudasphondylia* species were calculated by *p*-distance with MEGA XI (Tamura et al. 2021) and the divergence time was assessed with the divergent rates of 2.0 and 2.3% pairwise sequence divergence per million years according to DeSalle et al. (1987) and Brower (1994).

For the phylogeny, we evaluate the saturation of codon substitutions in order to obtain greater phylogenetic signal. The analyzed *COI* dataset were evaluated for transition and transversion substitutions by DAMBE 7 (Xia 2018). As result, the first codon and first plus second codons dataset were analyzed. The best model (T93+G) was selected for phylogenetic analysis using MEGA XI (Tamura et al. 2021) with Maximum likelihood method. Bootstrap replication was set as 1000.

#### RESULTS

#### TAXONOMY

# Order Diptera Linnaeus, 1758 Family Cecidomyiidae Newman, 1834 Tribe Asphondyliini Enderlein, 1914 Subtribe Asphondyliina Enderlein, 1914 Genus *Pseudasphondylia* Monzen, 1995

Pseudasphondylia Monzen 1955: 41. Type species: Pseudasphondylia rokuharensis Monzen, 1955.

Philadelphella Kovalev 1964: 440. Type species. Philadelphella philadelphi Kovalev, 1964.

The genus is known from China, Far-East Russia, India, Japan, New Caledonia, and Taiwan, and until now comprised 14 species (Gagné and Jaschhof 2021; Matsuda et al. 2021; Elsayed et al. 2023). A fifteenth

 Table 1. Pseudasphondylia specimens used in the molecular phylogenetic analysis, with information on host plant, locality, and DNA accession numbers

Gall midge	Host plant	Locality*	Taxon No. [Accession No.]
P. neolitseae	Neolitsea sericea	Fukuoka, JP	A348-350 [PV446537-39]
P. hooki sp. n.	N. acuminatissima	Mt. Dahan, Pingtung, TW	A74,A77 [PV446529-30]
	N. daibuensis	Jinshuiying Trail 0.7K, Pingtung, TW	A354-356 [PV446540-42]
	N. konishii	Donggua Trial, Miaoli, TW	A180,A360 [PV446531,PV446543]
	N. parvigemma	Mt. Dahan forest Trial, Pingtung, TW	A257-258 [PV446532-33]
	N. sericea	Erziping trail, Taipei, TW	A247-249,A375-259 [PV446534-36,PV446544-46]
	N. variabillima	Mt. Beidongyan-Nantungyen 3.4K, Nantou, TW	A362-363 [PV446547-48]

\*TW and JP indicate Taiwan and Japan, respectively.

species is described below from Taiwan on *Neolitseae* species. The description, DNA, and host spectrum of the new species were provided below.

# Pseudasphondylia hooki sp. n. Lin, Tokuda & Yang

(Table 2; Figs. 3–4) urn:lsid:zoobank.org:act:716CE8F9-AF4D-42B5-81FF-882AF8940986

*Type materials: Holotype:*  $\diamond$  (on slide, NCHU), TAIWAN: Taipei City, Mt. Yang-Ming National Park, gall collected on 17.ii.2011 and adult emerged on 23.ii.2011, ex. *Neolitsea sericea*, leg. SF Lin.

Paratypes: TAIWAN: Ex. Neolitsea *acuminatissima*: [Taipei City] 4  $\stackrel{\circ}{_{+}}$ , 4 pupal exuviae (on slides, NCHU), gall collected on 22.i.2013 and adult emerged on 26.i.2013, Mt. Yang-Ming National Park, leg. SF Lin; [Nantou Co.] 4  $\Diamond$ , 1  $\Diamond$ , 1  $\Diamond$ , 1  $\Diamond$ (on slides, NCHU), gall collected on 23.iii.2013 and adult emerged on 25-28.iii.2013, Mt. Living, leg. SF Lin; [Yilan Co.] 2 pupae (on slides, NCHU), 12.ii.2009, Taiwan Beech National Trail, Mt. Taiping, leg. TC Tang. Ex. *Neolitsea konishii*: [Taipei City] 4 , 9 , 3 pupae, 26 pupal exuviae (on slides, NCHU), gall collected on 17.ii.2011 and adult emerged on 24-26. ii.2011, Mt. Yang-Ming National Park, leg. SF Lin; 5 , 8 , 2 pupae, 6 pupal exuviae (on slide, NCHU), 11.iii.2011, Mt. Yang-Ming National Park, leg. SF Lin; 8  $\stackrel{\circ}{_{\sim}}$ , 12 pupal exuviae (on slides, NCHU), gall collected on 22.i.2013, adult emerged on 28.i.2013, Mt. Yang-Ming National Park, leg. SF Lin. Ex. Neolitsea sericea **[Taipei City]** 2  $\Diamond$ , 6  $\Diamond$ , 7 pupae, 50 pupal exuviae (on slides, NCHU), same data as holotype;  $1 \stackrel{\circ}{\uparrow}$ , 2 pupae, 9 pupal exuviae (on slides, NCHU), gall collected on 18.iii.2011 and adult emerged on 20.iii.2011, Mt. Yang-Ming National Park, leg. SF Lin; 2 mature larvae (on slides, NCHU), 30.xii.2013, Mt. Yang-Ming National Park, leg. SF Lin; 5 &, 1 pupa (in EtOH, NCHU), 11.ii.2001, Mt. Yang-Ming National Park, leg. LH Liao, MM Yang; 10 & (in EtOH, NMNS), gall collected on 29.i.2024 and adult emerged on 4~5.ii.2024, Mt.

Yang-Ming National Park, leg. SF Lin. Ex. *Neolitsea parvigemma*: [Pingtung Co.] 8 mature larvae (six in EtOH, two on slide, NCHU), 17.ii.2017, Dahan forest rd. leg. SF Lin. Ex. *Neolitsea variabillima*: [Nantou Co.] 1 mature larva (in EtOH, TFRI), 18.i.2010, Mt. Peitungyen-Nantungyen 3.4K, leg. GS Tung.

Adult: Head. Eye bridge 3–4 facets long. Frontoclypeal setal count as in table 2. Labella hemispherical in lateral view, with setae. Palpus threesegmented (Fig. 2A), first segment globose, 25–30  $\mu$ m long, with 2–4 setae; second 2.0 times as long as first, with 4–8 setae and third 2.2 times as long as first, with 7–10 setae. Twelve flagellomeres, the last three equal in length in male (Fig. 2B). Female distal flagellomeres gradually shortened, flagellomere 11 subglobular and terminal one globular (Fig. 2C).

*Thorax*: Thoracic setal and scale counts as in table 2. Legs with dense blackish scales; first tarsomeres of all legs with apicoventral spur; tarsal claws bent after midlength on all legs; pulvilli shorter than claws; empodia slightly shorter than claws on all legs (Fig. 2D–E). Wing 3.1–4.0 mm long, 2.0–2.4 times as long as wide in male; 3.2-3.4 mm, 2.3 times as long as wide in female. R<sub>5</sub> joining costa posterior to wing apex (Fig. 2F–G).

*Male abdomen*: Tergites I–VII rectangular, covered with scales on medium part, with a few lateral setae; tergites I–VI with 1–2 posterior row of setae; tergite VII with few setae on posterior part; sternites II–VII with anterior pair of lateral setae, cover with scattered setae and scales, sternites I–VI with three to four posterior row of setae; tergite VII with three to four posterior row of setae; tergite VII with anterior pair sternites VII with three to four posterior row of setae; tergite VII covered with scattered setae and few scales.

*Terminalia* (Fig. 2H): cerci forming a pair of lobes, each lobe with a few apical setae; hypoproct bilobed, deeply separated, each lobe with two apical setae; gonostylus suboval, with two separate sclerotized teeth cover with setae; gonocoxite elongate, cover with setae dorsally and ventrally; mediobasal lobe of gonocoxite present; aedeagus laterally sclerotized in basal part, distally tapering.

Table 2. Frontoclypeal and thoracic setal counts of Pseudasphondylia hooki sp. n.

Specimens		Male			Female		
	n	$Mean \pm SD$	Range	п	$Mean \pm SD$	Range	
Frontoclypeal setae	3	$29.3\pm4.7$	24–33	2	$41.5\pm0.7$	41-42	
Anterior dorsolateral setae	4	$66.5\pm6.4$	63-76	4	$56.3\pm5.7$	49-63	
Posterior dorsolateral setae	3	$65.3\pm7.0$	58-72	4	$64.3\pm5.6$	59-72	
Mesopleural scales	5	$14.6\pm1.8$	12-17	4	$12.0 \pm 1.8$	10-14	
Mesepimeral setae	6	$46.5\pm9.4$	30–55	4	$35.5\pm4.1$	32–40	



**Fig. 2.** *Pseudasphondylia hooki* sp. n. (A) Male head (ventral view) (B) Male antenna (8–12 segment). (C) Female antenna (8–12 fragellomeres). (D) Male 5th tarsomere. (E) Female 5th tarsomere. (F) Male wing. (G) Female wing. (H) Male genitalia. (I) Female abdomen. (J) Pupal abdomen. Scale bars: A-C = 0.03 mm; D-E = 0.2 mm; F-G = 1 mm; H = 0.2 mm; I-J = 0.5 mm.

*Female abdomen*: First through seventh tergites and second through sixth sternites as in male. Seventh sternite 0.5–0.6 mm long, protrusible part of ovipositor 1.3–1.7 mm long (Fig. 21), 2.5–3.1 times as long as the length of seventh sternite.

*Pupa*: Body length 2.7–3.0 mm, pupal skin not pigmented except for antennal horns. Antennal horn 280–320 μm long, triangular, lateral margin without irregular serration (Fig. 3); cephalic papilla with seta, 40–65 μm long; frons without horns; two pairs of facial and three pairs lateral facial papillae present; prothoracic spiracle (Fig. 3) 180–210 μm long; second to sixth abdominal spiracles  $50-70 \ \mu m$  long; second to seventh abdominal segments with 4 to 5 transverse rows of spines (Fig. 2J); eighth abdominal segments with 3 to 4 transverse rows of spines; eight dorsal papillae on first to the seventh abdominal segments, most outer and second inner pair papillae with seta; two dorsal papillae on eighth abdominal segment, each with seta; pleural papilla present on each side, with seta.

*Mature Larva*: Body color in life yellow, length 2.0–2.3 mm. Second antennal segment short, conical; cervical papillae without seta. Sternal spatula 330–370 µm long, anteriorly with two lobes (Fig. 3);



# 0.02

**Fig. 3.** Phylogenetic tree based on the 1<sup>st</sup> codon positions of *COI* (T93+G) and morphological differences of *Neolitsea*-associated *Pseudasphondylia* gall midge, *P. hooki* sp. n. (left) and *P. neolitseae* (right). The bootstraps (%) of Maximum-Likelihood inferences were shown beside nodes (1<sup>st</sup> codon/ $1^{st} + 2^{nd}$  codons). Score less than 60 were hidden.

four lateral papillae and a sternal papilla present on each side of all thoracic segments, each with seta; four dorsal papillae on all thoracic and first to seventh abdominal segments, two dorsal papillae on eighth abdominal segment, each with seta; pleural papilla present on each side, with seta; 2 asetose terminal papillae present; anal papillae not apparent.

## Distribution: Taiwan.

Gall and Host: Monothalamus, spherical gall on the leaves of six Neolitsea species, N. acuminatissima, N. daibuensis, N. konishii, N. parvigemma, N. sericea, and N. variabillima. Gall development and morphology are shown in Liang et al. (1999) and Tung et al. (2018). A total of 1059 galls of P. hooki were examined, including 225 galls on N. acuminatissima, 62 on N. daibuensis, 98 on N. konishii, 90 on N. parvigemma, and 500 on N. variabillima. All of them occurred on the abaxial side of the host leaf (Table 3).

*Biological notes*: This species is fundamentally univoltine. Adults emerge directly from galls in February and females lay eggs possibly into new buds as in *P. neolitseae*. Larvae soon hatch and remain inside the host tissue as the first instar until October. Liang et al. (1999) mentioned the gall started to grow from August and reached the maturation phase in October. Subsequently, larvae within the galls undergo maturation in January and pupate during middle mid-January and early February. While Takasu and Yukawa (1984) reported the occurrence of extended diapause and a semivoltine life cycle in Japanese *P. neolitseae*, the presence of such individuals in *P. hooki* remains unconfirmed

*Remarks*: Male terminalia and larval features, sternal spatula with two lobes and cervical and ventral papillae lacking seta, of the new species are similar to those of *P. neolitseae*. Nevertheless, the new species is distinguishable from *P. neolitseae* by the deeper insersion of larval sternal spatula (Fig. 3), serrate lateral margin of the antennal horns (Fig. 3) and hookliked prothoracic spiracle (Fig. 3) in pupa, and the three-segmented palpus. In contrast to these features, *P. neolitseae* possesses sternal spatula with shallower insersion in larva, antennal horns with flat lateral margin and long prothoracic spiracle in pupa, and twosegmented palpus in adult.

# Species delimitation and divergence

A total of 20 *COI* sequences (~658 bp) of *Neolitsea*-associated *Pseudasphondylia* species were obtained. Among them, 17 *COI* sequences of *P. hooki* were obtained from six host plants in Taiwan and three from *P. neolitseae* on *N. sericea* in Japan (Table 1). Excluding the three outgroups, the ASAP revealed that *P. neolitseae* formed one entity under all asap-score modes, whereas *P. hooki* formed one to 11 entities with different asap-score modes. The ASAP branch nodes of the two species did not support their distinction (P > 0.1) (Fig. 4). The *COI* genetic distance is 0.7% within *P. hooki* sp. n., 0% within *P. neolitseae*, while 2.7% (2.5–

Host	Locality	Abaxial side	Adaxial side
N. acuminatissima	Mt. Beidongyan, Nantou	27	0
	Mt. Liying, Nantou	60	0
	Cika Cabin, Taichung	90	0
	Dasyueshan forest rd.	48	0
N. daibuensis	Jinshuiying Trail, Pingtung	62	0
N. konishii	Erziping Trail, New Taipei	48	0
	Sanyi, Maioli	4	0
	Huisun forest area, Nantou	16	0
	Mt. Dongmao, Taichung	25	0
	Dasyueshan forest rd., Taichung	5	0
N. parvigemma	Jinshuiying Trail, Pingtung	90	0
N. variabillima	Mt. Beidongyan, Nantou	23	0
	Mt. Baimao, Taichung	2	0
	Lianhuachi Research Center, Nantou	2	0
	Dasyueshan forest rd., Taichung	57	0
N. sericea	Erziping Trail, New Taipei	500	0

Table 3. Galled position of Pseudasphondylia hooki sp. n. on Neolitsea leaves

3.1%) between the two species. The divergence time between them was estimated at around 1.2–1.3 mya.

## Molecular phylogeny

The comparison of the transition and transversion frequencies of our two dataset revealed that the 1<sup>st</sup> codon and  $1^{st} + 2^{nd}$  codons did not reach saturation. In contrast, saturation was observed in other datasets, including those containing the 2<sup>nd</sup>, 3rd, and all codon positions (Fig. 5). Therefore, a total of 202 positions from the  $1^{st}$  codon and 404 positions from the  $1^{st} + 2^{nd}$ codons were used for further phylogenetic analysis. As a result, phylogenetic trees form both datasets shared similar topologies but some branches weakly supported in the tree reconstructed from the  $1^{st} + 2^{nd}$  codons. In the present study, we showed the tree based on 1<sup>st</sup> codon dataset as it most clearly illustrated the phylogenetic relationship. The phylogenetic tree supported the monophyly of Neolitsea-associated Pseudasphondylia species (bootstrap value: 90; Fig. 3), as well as of P. hooki lineage (bootstrap value: 79) and P. neolitseae lineage (bootstrap value: 93). In the P. hooki clade, individuals associated with N. sericea situated at the basal part and formed a paraphyletic group with respective a clade of P. hooki individuals on the other hosts (bootstrap value: 64). Then, P. hooki individuals on N. daibuensis formed a subclade (bootstrap value: 63) and situated at the most terminal part.

## DISCUSSION

The gall shapes together with host species of cecidomyiids are useful features for researchers to recognize cecidomyiid congeners in adjacent unexplored areas. In East Asia, P. elaeocarpi associated with Elaeocarpus sylvestris (Elaeocarpaceae) and inducing finger-shaped leaf galls in both Taiwan and Japan (Lin et al. 2022), and Bruggmanniella cinnamomi Tokuda & Yukawa inducing swollen stem galls on Cinnamomum yabunikkei (= C. japonica) in Japan and on Cinnamomum insulari-montanum in Taiwan (Lin et al. 2020b). However, similar ecological features may represent in distinct cecidomyiid species. For example, the shape of swollen stem galls of Taiwanese Bruggmenniella turoguei Lin, Yang, & Tokuda on *Cinnamomoum osmophloeum* is similar to Japanese B. cinnamomi on C. yabunikkei (Lin et al. 2020b). In the present study, spherical leaf galls on six Taiwanese Neolitsea species were all induced by P. hooki. This suggests the necessity of adopting the integrative taxonomic approach, in particular for galling cecidomyiid inducing similar galls and associated with related hosts in adjacent areas. Besides, gall morphospecies are used to evaluate the richness of galling insects in many studies (Yang et al. 2000; Tung et al. 2006; Pan et al. 2018). The case of P. hooki highlights the asymmetry of cecidomyiid species and gall morphospecies richness. In Taiwan, several Asphondyliina species are oligophagous and/or display



Fig. 4. Assemble Species by Automatic Partitioning (ASAP) analyses of *Neolitsea*-associated *Pseudasphondylia* gall midges based on mitochondrial partial *COI* region. Colors represent unique partitions. Gray and Yellow dots of branches refer to *P* value incalculable and greater than 0.1, respectively.

gall polymorphism (Pan et al. 2015; Lin et al. 2020a b; present study). These result in an overestimation of cecidomyiid richness based on host species and gall morphology. The further taxonomic efforts of Taiwanese Asphondyliina are needed to realize the actual cecidomyiid diversity. Additionally, these study systems are suitable materials to explore the divergence mode of galling cecidomyiids at the species level.

## Species recognition and divergence

The mtDNA marker, the partial *COI* region, is a useful tool for the recognition of cecidomyiid species and the study of their evolutionary history. Tokuda et al. (2004) clarified the host spectrum and biogeography of *Ilex*-associated *S. sasakii* and *S. soyogo* (mentioned as *Asteralobia* species at that time) in Japan; Lin et al. (2020b) confirmed the species identify and possible evolutionary history of four *Cinnamomum*-associated



Fig. 5. Saturation plots of transition (blue line) and transversion (green line) rates against F84 distance.

*Bruggmanniella* species in the East Asian Arc. In the present study, the ASAP species delimitation (Fig. 4) showed the distinct relationship of *P. hooki* and *P. neolitseae*, which was also supported by morphological evidence and our ML tree (Fig. 3).

Our phylogenetic analysis of Neolitsea-associated Pseudasphondylia species highlighted the inter- and intraspecific divergence of cecidomyiids involving island biogeography and host expansion. In the biogeography of Neolitsea-associated Pseudasphondylia species, the divergence time (1.2–1.3 mya) of these two species corresponds to a later period of separation between Taiwan and Japan in the late Pliocene (Kimura 1996; Osozawa et al. 2012). This divergence timing is similar to that of Taiwanese and Japanese population of P. elaeocarpi (Lin et al. 2022). As to host expansion, N. sericea can be regarded as the primitive host of Neolitsea-associated Pseudasphondylia species based on the host distribution in the phylogeny (Fig. 3). The insular host expansion of P. hooki can be inferred together with the plant range and the dispersal route from north to south in Taiwan. The basal clade of P. hooki associated with N. sericea, which is mainly found in northern Taiwan and the terminal clade with N. daibuensis, which are endemic to southern Taiwan (Lu et al. 2000).

In P. neolitseae, galls were induced both on the abaxial and adaxial sides of leaf (mentioned as the lower and upper types in Mishima and Yukawa 2007) and these types were distributed parapatrically in Honshu (Kanto Region) and Kyushu (Sunose 1981; Mishima and Yukawa 2007). Host ploidy level is not related to the dimorphism (Mishima and Yukawa 2007), but, based on a transplanting experiment, gall midge genotype seems to be involved in it (Yukawa and Takahashi 2017). Unlike P. neolitseae representing gall dimorphism, all galls of P. hooki occur only on the abaxial side (Table 3), this type is assumed to be the primitive gall type in Neolitsea-associated Pseudasphondylia lineage relying on the parsimonic evolution in the gall habitat. More DNA data of P. neolitseae from Kyushu and Honshu (Kanto region) are needed to explore the evolutionary transition of gall type in P. neolitseae, as well their possible divergence route.

## CONCLUSIONS

The Taiwanese *Pseudasphondylia* species associated with *Neolitsea* is morphologically distinct from Japanese congener *P. neolitseae*, and the result is supported by the species delimitation using mtDNA *COI* region and ASAP analysis. These resulted in the formal description of a new species *P. hooki*. The sistership of *P. hooki* and *P. neolitseae* may indicate their recent speciation via geographical isolation. Moreover, *N. sericea* is assumed to be the primitive host of *Neolitsea*associated *Pseudasphondylia* species. Host expansion and insular dispersal route of *P. hooki* have been inferred, with evidence indicating a movement from north, associated with *N. sericea*, to south, associated with *N. daibuensis*, in Taiwan as supported by the phylogenetic relationship.

Acknowledgments: We thank the National Science Council of Taiwan (MOST 104-2313-B-005-015-MY2 and NSC 97-2313-B-005-033-MY3), Taiwan Entomological Society (Professor Sato Taxonomy Scholarship) and Interchange Association, Japan (Japan-Taiwan Joint Research Program) for their funding support. We would like to express our gratitude to Dr. Raymond J. Gagné for his valuable comments on this manuscript, and to Dr. Cheng-Lung Tsai and Dr. Wen-Bin Yeh for their assistance with the molecular work. We grateful to the Forestry and Nature Conservation Agency of Taiwan and Yangmingshan National Park Headquarters for issuing the collection permission.

**Authors' contributions:** SFL, TGS, PLY, MT, and MMY collected the specimens and ecological data; SFL finished the specimen's preparation, illustrations, description, and molecular work; MT and MMY got funds and supervise the work; all authors wrote the manuscript, approved the final manuscript and consent to its publication.

**Competing interests:** All authors declare that they have no competing interests.

**Availability of data and materials:** DNA sequences generated in the study have been deposited into the DNA Data Bank of Japan (DDBJ) database.

**Consent for publication:** All the authors consent to the publication of this manuscript.

**Ethics approval consent to participate:** This research followed the guidelines specified by the research permits from the Forestry Bureau, Council of Agriculture in Taiwan.

## REFERENCES

Brower AVZ. 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. Proc Natl Acad Sci USA **91:**6491–6495. doi:10.1073/pnas.91.14.6491.

- Cameron SL, Lambkin CL, Barker SC, Whiting MF. 2007. A mitochondrial genome phylogeny of Diptera: whole genome sequences resolve relationships over broad timescales with high precision. Syst Entomol **32:**40–59. doi:10.1111/j.1365-3113.2006.00355.x.
- DeSalle R, Freeman T, Prager EM, Wilson AC. 1987. Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. J Mol Evol 26:157–164. doi:10.1007/BF02111289.
- Elsayed AK, Yukawa J, Tokuda M. 2018. A taxonomic revision and molecular phylogeny of the eastern Palearctic species of the genera *Schizomyia* Kieffer and *Asteralobia* Kovalev (Diptera: Cecidomyiidae: Asphondyliini), with descriptions of five new species of *Schizomyia* from Japan. Zookeys **808:**123–160. doi:10.3897/zookeys.808.29679.
- Elsayed AK, Nemoto S, Tokuda M. 2023. *Pseudasphondylia ishigakiensis*, a new gall midge species (Diptera: Cecidomyiidae) from Japan developing in flower buds of *Melicope triphylla* (Rutaceae), with notes on its life history. J Asia-Pacific Entomol **26:**102107. doi:10.1016/j.aspen.2023.102107.
- Enderlein G. 1914. Ord. Diptera. Fliegen (Zweiflügler). In: Brohmer P (ed) Fauna von Deutschland. Ein Bestimmungsbuch unserer heimischen Tierwelt. Quelle & Meyer, Leipzig, pp. 272–334.
- Gagné RJ. 1994. The gall midges of the Neotropical region. Cornell University Press, Ithaca, NY.
- Gagné RJ. 2018. Key to adults of North American genera of the subfamily Cecidomyiinae (Diptera: Cecidomyiidae). Zootaxa 4392:401–457. doi:10.11646/zootaxa.4392.3.1.
- Gagné RJ, Jaschhof M. 2021. A catalog of the Cecidomyiidae (Diptera) of the world. 5th Edition. Digital, 813 pp. Available at: https://www.ars.usda.gov/ARSUserFiles/80420580/Gagne\_ Jaschhof 2021 World Cat 5th Ed.pdf. Accessed 18 Apr. 2021.
- Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symp Ser **41**:95–98.
- Kimura M. 1996. Quaternary paleogeography of the Ryukyu Arc. Geogr J 105:259–285. doi:10.5026/jgeography.105.3\_259. (in Japanese with English abstract)
- Kovalev OV. 1964. A review of the gall midges (Diptera, Itonididae) of the extreme south of the Soviet Far East. I. The supertribe Asphondylidi. Entomological Review **43**:215–228.
- Liang LM, Yang SY, Yang JZ, Chen MY. 1999. Development of the Insect-induced Galls of *Neolitsea Aciculata* var. variabillima and *Litsea acuminata* in Guandaushi Forest Ecosystem. Bot Bull of Acad Sinica 43:31–35. (in Chinese)
- Lin SF, Tokuda M, Tung GS, Pan LY, Kim W et al. 2022. Biogeography and ecological differentiation of *Pseudasphondylia* gall midges (Diptera: Cecidomyiidae) distributed in Taiwan and Japan, with description of a new species *P. kiwiphila* sp. nov. and the southernmost record of *P. elaeocarpi*. Zool Stud **61**:39. doi:10.6620/ZS.2022.61-39.
- Lin SF, Tokuda M, Yang MM. 2019. The first record of genus Bruggmanniella (Diptera: Cecidomyiidae) from Taiwan with description of a new species inducing bud galls on Neolitsea parvigemma (Lauraceae). J Asia Pac Entomol 22:203–207. doi:10.1016/j.aspen.2018.12.012.
- Lin SF, Tokuda M, Yang MM. 2020a. Leaf gall polymorphism and molecular phylogeny of a new *Bruggmanniella* species (Diptera: Cecidomyiidae: Asphondyliini) associated with *Litsea acuminata* (Laruaceae) in Taiwan, with ecological comparisons and a species description. Entomol Sci **23:**10–22. doi:10.1111/ens.12391.
- Lin SF, Yang MM, Tokuda M. 2020b. Molecular phylogeny revealing the single origin of *Cinnamomum*-associated *Bruggmanniella* (Diptera: Cecidomyiidae) in Asia, with descriptions of three new and one newly recorded species from Taiwan. Zool Stud **59:**66. doi:10.6620/ZS.2020.59-66.

- Lin SF, Tokuda M, Tung GS, Pan LY, Kim W et al. 2022. Biogeography and ecological differentiation of *Pseudasphondylia* gall midges (Diptera: Cecidomyiidae) distributed in Taiwan and Japan, with description of a new species *P. kiwiphila* sp. nov. and the southernmost record of *P. elaeocarpi*. Zool Stud **61:3**9. doi:10.6620/ZS.2022.61-39.
- Linnaeus C. 1758. Systema Naturae per Regna Tria Naturae. Edition 10, vol. I. Stockholm, Sweden.
- Lu FY, Ou CH, Chen YC, Chi YS, Lu KC. 2000. Trees of Taiwan. vol 1. – Department of Forestry, National Chung Hsing University, Taichung, Taiwan.
- Matsuda H, Elsayed AK, Kim W, Yamauchi S, Libra M et al. 2021. A new species of *Pseudasphondylia* (Diptera: Cecidomyiidae) associated with *Magnolia kobus* DC. var. *borealis* Sarg. (Magnoliaceae) in Japan. Biodiversity Data Journal 9:e68016. doi:10.3897/BDJ.9.e68016.
- Mishima M, Yukawa J. 2007. Dimorphism of leaf galls induced by *Pseudasphondylia neolitseae* (Diptera: Cecidomyiidae) on *Neolitsea sericea* (Lauraceae) and their distributional patterns in Kyushu, Japan. Bulletin of the Kyushu University Museum 5:57–64.
- Monzen K. 1955. Some Japanese gallmidges with the descriptions of known and new genera and species (II). Annu Rep Gakugei Fac Iwate Univ 9:34–48.
- Newman E. 1834. Attempted division of British insects into natural orders. Entomol Mag **2:**379–431.
- Osozawa S, Shinjo R, Armid A, Watanabe Y, Horiguchi T et al. 2012. Palaeogeographic reconstruction of the 1.55 Ma synchronous isolation of the Ryukyu Islands, Japan, and Taiwan and inflow of the Kuroshio warm current. Int Geol Rev 54:1369–1388. doi:10. 1080/00206814.2011.639954.
- Paik JC, Yukawa J, Uechi N, Sato S, Ganaha T. 2004. Gall-inducing species of the family Cecidomyiidae (Diptera) recorded from the Korean peninsula and surrounding islands, in comparison with the gall-midge fauna of Japan. Esakia 44:57–66. doi:10.5109/2682.
- Pan LY, Chiang TC, Weng YC, Chen WN, Hsiao SC et al. 2015. 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. Zootaxa 3955:371–388. doi:10.11646/zootaxa.3955.3.5.
- Pan LY, Liu J, Yang MM, Tung GS. 2018. Diversity of arthropod galls in Taiwan. Formosan Entomol 38:42–55. doi:10.6662/ TESFE.2018018.
- Puillandre N, Brouillet S, Achaz G. 2021. ASAP: assemble species by automatic partitioning. Mol Ecol Resour 21:609–620. doi:10.1111/1755-0998.13281.
- Sunose T. 1981. A study of biogeography on the Izu Islands with notes on the distribution of gall midges. Panmixia **4:**8–12. (in Japanese)
- Takasu K, Yukawa J. 1984. Two-year life history of the neolitsea leaf gall midge, *Pseudasphondylia neolitseae* Yukawa (Diptera, Cecidomyiidae). Kontyû **52:**596–604.
- Tamura K, Stecher G, Kumar S. 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol 38:3022–3027. doi:10.1093/molbev/msab120.
- Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res **22:**4673– 4680. doi:10.1093/nar/22.22.4673.
- Tokuda M. 2004. Illiciomyia Tokuda, a new genus for Illiciomyia yukawai sp. n. (Diptera: Cecidomyiidae: Asphondyliini) inducing leaf galls on Illicium anisatum (Illiciaceae) in Japan. Esakia 44:1–11. doi:10.5109/2678.

- Tokuda M, Tabuchi K, Yukawa J, Amano H. 2004. Inter- and intraspecific comparisons between *Asteralobia* gall midges (Diptera: Cecidomyiidae) causing axillary bud galls on *Ilex* species (Aquifoliaceae): species identification, host range, and mode of speciation. Ann Entomol Soc Am **97:**957–970. doi:10.1603/0013-8746(2004)097[0957:IAICBA]2.0.CO;2.
- Tokuda M, Yukawa J. 2005. Two new and three known Japanese species of genus *Pseudasphondylia* Monzen (Diptera: Cecidomyiidae: Asphondyliini) and their life history strategies. Ann Entomol Soc Am **98**:259–272. doi:10.1603/0013-8746(2005)098[0259:TNATKJ]2.0.CO;2.
- Tung GS, Yang MM, Lin SF, Tang CT, Liao YC et al. 2018. Let's Gall Taiwan – A guidebook on insect galls. Forestry Bureau C. O. A, Taipei, 247 pp.

- Tung GS, Yang PS, Yang MM. 2006. Pattern analysis of galling hostplants in Taiwan. Taiwan J Forest Sci 21:205–214. doi:10.7075/ TJFS.200606.0205. (in Chinese)
- Xia X. 2018. DAMBE7: New and improved tools for data analysis in molecular biology and evolution. Mol Biol Evol **35**:1550–1552. doi:10.1093/molbev/msy073.
- Yang SY, Chen MY, Yang JT. 2000. Plant galls in Taiwan. Taiwan Counsil of Agriculture, Taipei, 127 pp. (in Chinese)
- Yukawa J. 1974. Descriptions of new Japanese gall midges (Diptera, Cecidomyiidae, Asphondyliidi) causing leaf galls on Lauraceae. Kontyû 42:293–304.
- Yukawa J, Takahashi K. 2017. Gall polymorphism in *Pseudasphondyia* neolotseae; the 42nd year of the host exchange experiment. Pulex 96:718–720. (in Japanese)