Is a Gall an Extended Phenotype of the Inducing Insect? A Comparative Study of Selected Morphological and Physiological Traits of Leaf and Stem Galls on *Machilus thunbergii* (Lauraceae) Induced by Five Species of *Daphnephila* (Diptera: Cecidomyiidae) in Northeastern Taiwan

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Is a Gall an Extended Phenotype of the Inducing Insect? A Comparative Study of Selected Morphological and Physiological Traits of Leaf and Stem Galls on *Machilus thunbergii* (Lauraceae) Induced by Five Species of *Daphnephila* (Diptera: Cecidomyiidae) in Northeastern Taiwan

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Mature galls induced by *Daphnephila truncicola*, *D. taiwanensis*, *D. sueyenae*, *D. stenocalia*, and *D. ornithocephala* on *Machilus thunbergii* in northern Taiwan were examined to verify the dictum that the morphology of galls is an expression of the extended phenotype of the respective gall-inducing insect. Based on their length-width ratio, the materials were grouped into either fleshy (those induced by *D. taiwanensis* and *D. sueyenae*) or slim galls (those induced by *D. truncicola*, *D. stenocalia*, and *D. ornithocephala*). Stem galls induced by *D. truncicola* showed an energy level of 0.0178 kJ/g. Among leaf galls, the greatest energy level was in the one induced by *D. stenocalia* (0.0193 kJ/g), followed by *D. sueyenae* (0.0192 kJ/g), *D. taiwanensis* (0.0189 kJ/g), and *D. ornithocephala* (0.0160 kJ/g). The numbers of reserve and nutritive cell layers in galls were greater in the stem galls induced by *D. truncicola*, similar to those in the fleshy leaf galls, than in the slim leaf galls. Based on the fungal taxa isolated from the larval chambers and considering the similarities and divergences among gall characteristics, the galls induced by *D. truncicola* and *D. taiwanensis* clustered into one, whereas those of *D. sueyenae* aligned with the '*D. stenocalia–D. ornithocephala*' cluster. The present study verified that shapes, structure, nutritive tissues, energy levels, and multiple coexisting fungal taxa within galls reinforce that they are extended phenotypes of the respective gall-inducing *Daphnephila* species and they represent adaptive evolution of *Daphnephila* on *M. thunbergii*.

Key words: *Daphnephila*, nutrition sink ability, *Machilus thunbergii*, gall shape, fungi

INTRODUCTION

A majority of gall-inducing insects display a high level of specificity to plants (Raman, 1996; Abrahamson et al., 1998, 2003; Raman et al., 2005) and each gall-inducing insect species generally induces a gall of specific morphology (Raman, 2011). Measurements of congruence and departures from their levels of specificity enable interpretations of the evolution of gall-inducing insects (Joy and Crespi, 2007). Biological and biogeographical treatments (Roskam, 1985, 1992) indicate that the galls induced by the Cecidomyiidae serve as useful model systems to make such measurements in the context of evolution of phytophagy and cecidogeny (Price, 2005; Yukawa et al., 2005). Many congeneric Cecidomyiidae induce galls of varied morphologies either on the same or on closely related species (Skuhravá et al., 1984; Yukawa et al., 2005; Raman, 2007; Tokuda et al., 2008). Among the gall-inducing Cecidomyiidae (Gagné, 1989; Skuhravá, 2006), the levels of specificity of *Daphnephila* to species of Lauraceae are striking (Tokuda and Yukawa, 2007).

At least 30 species of *Daphnephila* (Asphondyliini: Asphondyliina) are suspected to induce galls on the eastern Palearctic and Oriental species of *Machilus* and *Persea* (the *Persea* group of Lauraceae, *sensu* Chanderbali et al., 2001; Li et al., 2011). Only nine of these have been named: three from India, one from Japan, and five from Taiwan (Tokuda and Yukawa, 2007). Available biological information reveals

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that the second-stage larvae of the Japanese populations of *D. machilicola* overwinter within galls on *Machilus thunbergii*, and turn into third-stage larvae in the following spring (Yukawa, 1974). The species of *Daphnephila* have adopted type-IIA life-history strategy which was defined by Yukawa (1987) and was suggested to live a longer period than the other type-IIA gall midges (Tokuda et al., 2008). Historically *Daphnephila* originated in tropical south Asia and dispersed with *Machilus* through Taiwan to Japan (Tokuda and Yukawa, 2007; Tokuda et al., 2008). All five Taiwanese species induced galls of distinct morphologies on *M. thunbergii*, which sympatrically distributed in northeastern Taiwan (Tokuda et al., 2008). In addition to inducing galls of characteristic shapes, the Taiwanese species of *Daphnephila* are associated with fungi, a trait common in many of the Asphondyliini (Gagné, 1994; Roskam, 2005; Rohfritsch, 2008). Molecular phylogenetic analysis reveal that the stem-gall inducing insect *D. truncicola* occurred at an ancestral position relative to the leaf-gall inducing Ceciodmyiidae, viz., *D. taiwanensis*, *D. sueyenae*, *D. stenocalia*, and *D. ornithocephala*, whereas the phylogenetic relationships among *D. sueyenae*, *D. stenocalia*, and *D. ornithocephala* were not clear (Tokuda et al., 2008).

In the present study, we applied quantitative and qualitative measurements to compare 'mature' galls seeking answers to the following questions: Is the morphology of stem and leaf galls on *M. thunbergii* an expression of extended phenotype (*sensu* Dawkins, 1982; Weis et al., 1988) of the respective species of *Daphnephila*? Do these morphological and physiological gall characteristics indicate adaptive radiation of *Daphnephila* on *M. thunbergii*? To answer these questions, we characterized morphometrics and epidermal characteristics, total-energy contents, nature of reserve and nutritive tissues, and identity and distribution of fungi in the selected galls.

MATERIALS AND METHODS

Materials

Approximately 1,000 apparently fully-grown galls induced by *D. truncicola, D. taiwanensis, D. sueyenae, D. stenocalia*, and *D. ornithocephala* on *M. thunbergii* were randomly collected in Yangmingshan National Park (23°37'–23°49'N; 120°07'–120°16'E) and Fushan Botanical Garden (24°45'–24°46'N; 121°34'–121°35'E) located at an elevation of ~1000 m in north-eastern Taiwan between 17 and 20 January 2008. The galls were collected in polyethylene bags, placed immediately in an ice chest, and transported to the Department of Entomology, National Chung Hsing University (NCHU), Taichung, Taiwan. At the laboratory, the samples were refrigerated at 5°C. Each gall was slit vertically with a razor blade to verify the developmental stage of the inhabiting cecidomyiid. Galls inhabited by either the third-stage larvae or the pupae (hereafter, 'mature galls') were used in this analysis. The galls with the first- and second-larval stages, and parasitized inhabitants were excluded.

Morphometrics

Dissected mature galls induced by *D. truncicola* (n = 86), *D. taiwanensis* (n = 90), *D. sueyenae* (n = 82), *D. stenocalia* (n = 96), and *D. ornithocephala* (n = 44) were excised at points of their connection with their respective host organs. For each excised gall (consisting of the slit portions), measurements of length, width at the widest point, thickness at the adaxial side of larval-chamber wall, and thickness of the abaxial side of larval-chamber wall were determined with a digital caliper (CD–6" CSX, Mitutoyo, Japan) and a scientific millimeter ruler, under a stereo-binocular microscope (MZ125, Leica, Hamburg, Germany; Fig. 1). After removing the inhabitant, the galls were weighed (BL120S, Sartorius, Germany) and the mass of larvae was measured using a microbalance (M2P, Sartorius, Germany). Volumes of galls were calculated by adding geometric volume measurements and deduction of that of the larval chamber, which was treated as a cylinder. For example, volumes of the galls of *D. truncicola* and *D. taiwanensis* parts were considered as a cone and hemisphere (Fig. 1) and added, minus the larval chamber value treated as a cylinder to obtain the final volume value. Gall and larval fresh mass data were analyzed using Genstat 10 (Payne et al., 2007). Canonical-variate analysis was used to examine differences among the determined variables, which were the identities of the gall-inducing insect taxa (Tokuda et al., 2008) and compared to obtain within-group variances and co-variances facilitating a data presentation that best described associations among inducing taxa of insects. Following the tests of significance of canonical-variate axes, the Mahalanobis–distance measurement was applied to determine distances among groups, based on canonical variate scores.

Light microscopy

Mature galls induced by *D. truncicola* (n = 6) on stems, and *D. taiwanensis* (n = 6), *D. sueyenae* (n = 6), *D. stenocalia* (n = 6), and *D. ornithocephala* (n = 6) on leaves were excised and used for light microscopy. These were fixed in formal–acetic–alcohol (FAA: 70% ethanol 90 ml, 40% formalin 5 ml, and glacial acetic acid 5 ml) for 72 h. The fixed tissues were then transferred to 70% ethanol and stored for 24 h, followed by 80, 90, 95, and 100% ethanol (changed every 24 h). The fixed materials were processed through customary techniques for embedding in paraffin wax using tertiary–butyl–alcohol series. Sections (10 μm) were cut on a rotary microtome (Histostat 820, Reichert, NY, USA) and mounted on glass slides in Canada balsam after deparaffinization, clearing, and staining with safranin–fast green combination following Ruzin (1999).

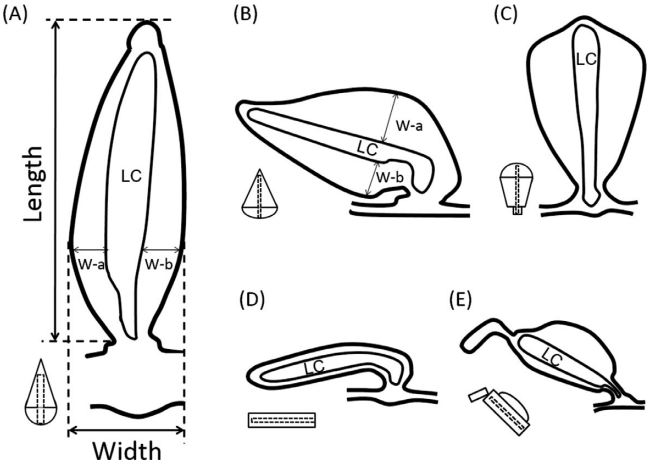

Fig. 1. Linear measurements of longitudinal view of mature *Daphnephila* galls. **(A)** *D. truncicola* gall attached to stem, **(B)** *D. taiwanensis* gall attached to leaf, **(C)** *D. sueyenae* gall attached to leaf, **(D)** *D. stenocalia* gall attached to leaf, **(E)** *D. ornithocephala* gall attached to leaf. 'W–a': abaxial side of the larval-chamber wall thickness, 'W–b': adaxial side of larval–chamber wall thickness, 'LC': larval chamber (figures not to scale). The subtending line illustrations along the lower left of each gall diagram illustrates the way gall volumes were calculated and the dashed line within them delineates the larval chamber.

Scanning electron microscopy

Mature galls induced by *D. truncicola* (*n* = 6), *D. taiwanensis* (*n* = 6), *D. sueyenae* (*n* = 6), *D. stenocalia* (*n* = 6), and *D. ornithocephala* (*n* = 6) were used for electron microscopy by fixing them in 50:50 formaldehyde–ethanol solution for 24 h. After fixation, materials were passed through an ascending-alcohol series (several times, retaining them for 2 h during each pass) and post–fixed in formaldehyde–dimethyl–acetate (FDA) solution for 24 h. Thereafter, materials were critical-point dried (HCP-2, Hitachi, Tokyo, Japan). Determined samples of gall tissues were mounted on SEM stubs and sputter-coated with gold (IB–2, Giko Engineering, Tokyo, Japan). Mounted specimens were viewed in an SEM (Inspect, FEI, Oregon, USA) at an acceleration voltage of 15 kV and photographs were obtained.

Calorimetry

Excised galls induced by *D. truncicola* (*n* = 47), *D. taiwanensis* (*n* = 21), *D. sueyenae* (*n* = 19), *D. stenocalia* (*n* = 63), and *D. ornithocephala* (*n* = 13), were partly slit vertically to extract the larvae. The excised gall tissues, without the larval material, induced by a specific taxon were pooled, packed in aluminum foils, and dried in an oven (3100–B, CHANNEL, New Taipei City, Taiwan) at 45°C until their mass values remained constant. Dried, pooled samples were weighed in a microbalance (M2P, Sartorius, Germany), hand-ground in an agate mortar, and subsequently fine-ground in an electric mortar (WIG-L-BUG, Bratte, USA). The mean-water content and mean-gall density were calculated using the following formulae:

$$\text{Mean water content (\%)} = \frac{\text{Mean fresh gall mass} - \text{Mean dry gall mass}}{\text{Mean gall fresh mass}} \times 100$$

$$\text{Mean gall density (mg/mm}^3\text{)} = \frac{\text{Mean dry gall mass}}{\text{Mean gall volume}}$$

An oxygen semi-microbomb calorimeter (OSMC) was used (PARR 1356, Parr Instrument Company, Illinois, USA). Because the pooled sample size was not amenable to the level of sensitivity of OSMC used, replications could be done only with samples of galls induced by *D. taiwanensis* (two replicates) and *D. truncicola* (eight replicates). Dried gall tissues were combusted (sample mass, 0.05–0.3 g) after standardization of the OSMC by combusting standard benzoic acid (five replicates).

Fungi

To identify the fungi associated with galls induced by the species of *Daphnephila*, refrigerated samples were dissected and the fungi were cultured in sterile Petri dishes with PDA (potato dextrose broth 39 g, agar 5.1 g, distilled water 1,000 ml) and CM (cornmeal agar 5.1 g, agar 1.5 g, and distilled water 300 ml). Each plate was labeled with the inducing-insect taxon and maintained at 25°C. Mycelia were harvested after 7–10 d and the fungi were determined by Chi-Yu Chen (Department of Plant Pathology, National Chung–Hsing University, Taiwan).

Similarity

We pooled the measured gall characteristics, viz., length, width at the widest point, thickness at the adaxial side of larval-chamber wall, thickness of the abaxial side of larval-chamber wall, gall color, gall surface texture, number of nutritive cell layers, energy levels, and coexisting fungal taxa, and analyzed with Gower's similarity coefficient by the statistical program PRIMER, version 6.1.5 (Clarke and Gorley, 2006).

RESULTS

Morphometrics

By measuring length-width ratio, shapes of galls, the galls could be grouped into fleshy (ratio ≤ 3) including *D. taiwanensis* and *D. sueyenae* and slim galls (ratio > 3) including *D. truncicola*, *D. stenocalia* and *D. ornithocephala* (Table 1). Among these, *D. stenocalia* was the smallest (6.33 mm^3) with mean-water content (48%), but bore the greatest ratio (41.53%) of mean larval chamber volume to the gall. Mean density was the lowest (0.08 mg/mm^3) in the galls of *D. ornithocephala*.

According to the canonical-variate analysis of the linear (length, diameter, adaxial gall-wall thickness of the larval chamber, abaxial gall-wall thickness of the larval chamber), and fresh-mass data of galls induced by the five species of *Daphnephila*, five discriminant functions and χ^2 tests indicated that their latent roots of within group variability (canonical variables, CV) were significant (Table 2).

Based on the standardized coefficients for the tested morphological features, the first canonical variable (CV–1) explained 56.76% of the discriminatory power of the model, whereas the second variable (CV–2) explained 39.57%. The morphometric clusters indicated that the fleshy galls induced by *D. sueyenae* and *D. taiwanensis* were mainly discriminated by CV–2, which had positive coefficients with respect to the slim galls induced by *D. ornithocephala*, *D. stenocalia*, and *D. truncicola* (Table 3).

The Mahalanobis–distance test was applied to compare morphometric divergence among gall populations. Within individual gall types, distances were of high significance among the leaf galls induced by *D. stenocalia*, *D. sueyenae,* and *D. taiwanensis*, and the stem gall induced by *D. truncicola* (Table 4). The basic gall configuration between *D. stenocalia* and *D. ornithocephala* were similar ($P < 0.01$), whereas the others were distinct ($P < 0.001$). The greatest distance among the leaf galls was between those induced by '*D. ornithocephala—D. stenocalia*' on the one hand and '*D. sueyenae—D. taiwanensis*' on the other; the stem galls induced by *D. truncicola* aligned closely with the '*D. ornithocephala—D. stenocalia*' cluster (Fig. 2).

Table 1. Morphometrics of unparasitized, mature *Daphnephila* galls.

Gall inducer	*n*	Gall-length (l) (mm)	Gall-width (w) (mm)	l—w ratio	Mean gall volume* (mm^3)	Proportion of mean larval chamber volume (%)	Mean gall density* (mg/mm^3)	Mean water content (%)
D. truncicola	83	13.96 ± 2.32	4.14 ± 1.03	3.48 ± 0.65	66.10	18.22	0.77	57.81
D. taiwanensis	60	8.64 ± 1.51	5.20 ± 1.30	1.75 ± 0.48	74.03	9.17	0.42	79.34
D. sueyenae	37	6.08 ± 1.32	3.97 ± 0.77	1.54 ± 0.24	30.88	12.93	0.53	73.48
D. stenocalia	55	7.69 ± 1.61	1.38 ± 0.26	5.76 ± 1.44	6.33	41.53	0.49	48.00
D. ornithocephala	23	10.44 ± 1.88	3.23 ± 0.68	3.36 ± 0.85	71.19	9.80	0.08	71.65

*The 'mean gall volume' and 'mean gall density' refer only to the plant tissue part of galls which subtracted the space of larval chamber.

Nature of epidermis

Mature galls induced by *D. truncicola* and *D. ornithocephala* were red, whereas those of *D. sueyenae* and *D. stenocalia* were green, and those induced by *D. taiwanensis* were red and green. The red galls remained red since the time of initiation but the sun-exposed parts of green galls, occasionally, turned red (Fig. 3C). All the five species galls presented a thick cuticle and lacked stomata (Fig. 3D). Galls of *D. truncicola* and *D. sueyenae* were smooth, and the others were quilted (Fig. 3E & F). Trichomes occurred only on the surface of *D. taiwanensis* (Fig. 3F).

Calorimetry

Energy levels in the galls induced by *D. stenocalia* and *D. sueyenae* were high with an average of 0.0193 and 0.0192 KJ/g, respectively. Galls of *D. ornithocephala* and *D. truncicola* had low levels of energy at 0.0160 and 0.0178 KJ/g, respectively. Galls induced by *D. taiwanensis* had an intermediate energy level of 0.0189 KJ/g. The energy per gall was the greatest in *D. truncicola* and the lowest in *D. stenocalia*. However, *D. stenocalia* had the highest energy per weight and per volume (mm^3).

Nutritive tissue

Nutrients for the gall-inducing Cecidomyiidae involve newly differentiated tissues that enclose the larvae, especially along the inner perimeter of the larval chambers. The galls induced by *Daphnephila* included parenchyma tissue and multiple layers of fungal mycelia. The stem gall induced by *D. truncicola* included 17.3 ± 1.9 layers of outer-lying reserve parenchyma, 5.3 ± 0.5 cell layers of active inner-lying nutritive parenchyma, and 5.3 ± 0.5 cell layers of fungi. The fleshy leaf-galls induced by *D. taiwanensis* included 27.7 ± 1.9 layers of outer-lying reserve parenchyma, 3.5 ± 0.5 layers of active inner-lying nutritive parenchyma, and 1.7 ± 0.5 layers of fungi. The leaf-galls induced by *D. sueyenae* included 27.0 ± 1.9 layers of active outer-lying reserve parenchyma, 3.3 ± 0.5 layers of active inner-lying nutritive parenchyma, and 3.0 ± 1.3 layers of fungi. The slim leaf galls induced by *D. stenocalia* included 6.5 ± 1.4 layers of active outer-lying reserve parenchyma, one layer of active inner-lying nutritive parenchyma, and 1.7 ± 0.5 layers of fungi. The slim leaf gall induced by *D. ornithocephala* contained 7.0 ± 1.1 outer-lying reserve parenchyma, one layer of active inner-lying nutritive parenchyma, and 1.3 ± 0.5 layer of fungus.

Fungi

All five galls on *M. thunbergii* hosted a range of fungal taxa. Among these, *Botryosphaeria dothidea* (anamorphs *Fusicoccum aesculi* and *Dichomera saubinetii* (Ascomycotina: Botryosphaeriales: Botryosphaeriaceae), more than one species of *Fusarium* (Ascomycota: Hypocreales: Nectriaceae), and one species of *Pestalotiopsis* (Ascomycota: Xylariales: Amphisphaeriaceae) (Table 5) occurred in all five galls.

Galls induced by *D. sueyenae*, *D. stenocalia*, and *D. ornithocephala* were generally associated with *Pestalotia* sp., whereas the galls of *D. stenocalia* and *D. sueyenae* included a species of *Phoma* (Ascomycota: Pleosporales). One species of *Colletortichum* (Ascomycota: Phyllosporales:

Table 2. Latent vector averages expressing within group variability.

Morphometric traits (Gall)	CV–1	CV–2
length	–1.5151	–0.0917
width	0.4462	–0.3305
adaxial larval chamber wall width	–0.4475	–0.1148
abaxial larval chamber wall width	–1.4039	–1.2582
fresh mass	0.0002	0.0000

Table 3. Means of canonical variates for the measured taxa.

Galls induced by	CV–1	CV–2
D. truncicola	1.619	–2.023
D. taiwanensis	–1.926	–0.767
D. sueyenae	–1.879	0.683
D. stenocalia	1.278	1.731
D. ornithocephala	1.571	0.787

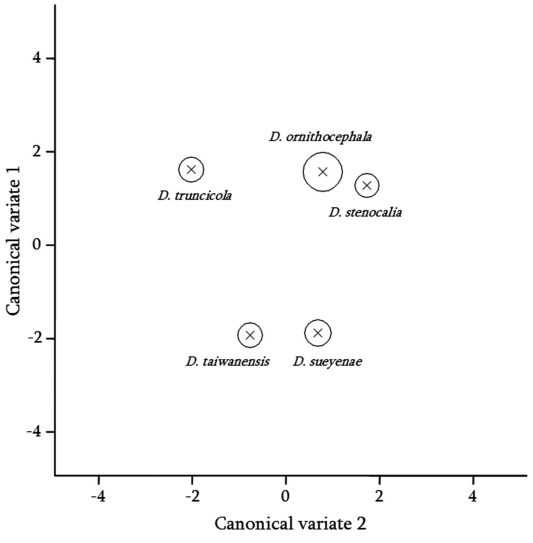

Fig. 2. Canonical variate—Mahalanobis distance analysis of the phenotypes of galls induced on stems by *Daphnephila truncicola* and on leaves by *D. taiwanensis*, *D. sueyenae*, *D. stenocalia*, and *D. ornithocephala*.

Table 4. Matrix of Mahalanobis distances[1].

Galls induced by	*D. truncicola*	*D. taiwanensis*	*D. sueyenae*	*D. stenocalia*	*D. ornithocephala*
D. truncicola	0				
D. taiwanensis	3.761***	0			
D. sueyenae	4.422***	3.827***	0		
D. stenocalia	3.769***	4.063***	3.451***	0	
D. ornithocephala	2.810***	1.451***	3.327***	0.988**	0

[1]Tested with Hotelling's test of significance: **$p < 0.01$; ***$p < 0.001$.

Table 5. The diversity of fungus species isolated from larval chamber of five *Daphnephila*.

Gall inducer	*Botryosphaeria dothidea*	*Fusarium* spp.	*Pestalotiopsis* sp.	*Pestalotia* sp.	*Phoma* sp.	*Colletortichum* sp.	*Cladosporium* sp.
D. truncicola	Y	Y	Y	N	N	N	N
D. taiwanensis	Y	Y	Y	N	N	N	Y
D. sueyenae	Y	Y	Y	Y	Y	Y	N
D. stenocalia	Y	Y	Y	Y	Y	N	N
D. ornithocephala	Y	Y	Y	Y	N	N	N

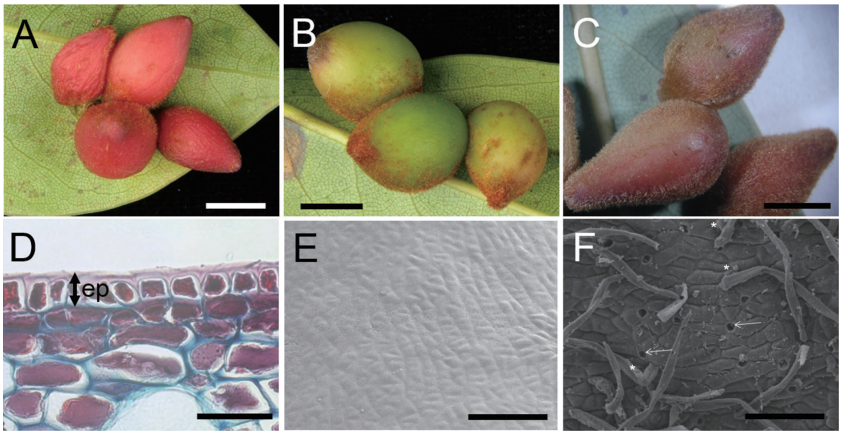

Fig. 3. **(A)** Galls of *Daphnephila taiwanensis* (red), bar = 5 mm, **(B)** Galls of *D. taiwanensis* (green), bar = 5 mm, **(C)** color-changing galls of *D. taiwanensis* (green to red), bar = 5 mm, **(D)** Wall of *D. truncicola* gall (red) with intensely cutinized epidermis, ep = epidermis layer, bar = 0.05 mm, **(E)** *D. sueyenae* gall, smooth surface without stomata contrasted to the other type shown in **(F)**, bar = 0.1 mm, **(F)** *D. taiwanensis* gall, quilted surface, * = trichomes, white arrow = residuals of abscised trichomes, bar = 0.1 mm.

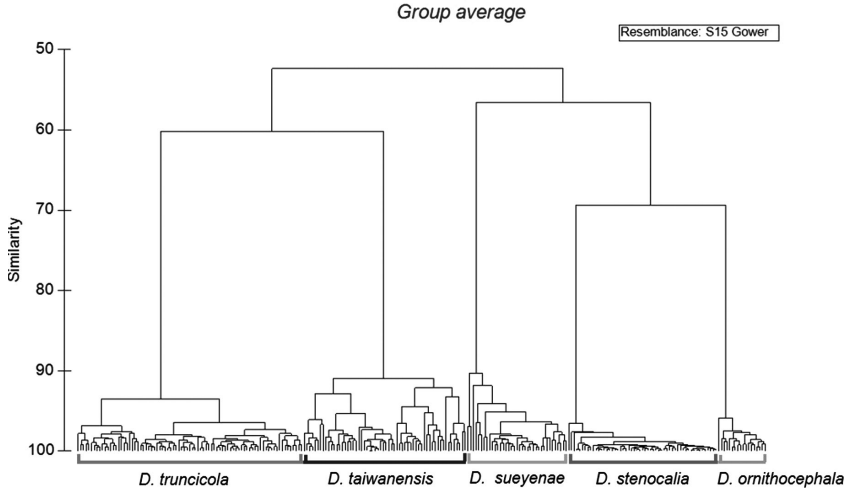

Fig. 4. Similarity of the galls induced by *Daphnephila* gall midges.

Phyllosporaceae) occurred in the galls of *D. sueyenae* and one species of *Cladosporium* (Ascomycota: Capnodiales: Davidiellaceae) occurred in the galls of *D. taiwanensis*.

Similarity

There were two major clusters in the similarity analysis (Fig. 4). The stem galls induced by *D. truncicola* were grouped with the leaf galls induced by *D. taiwanensis* and the other three species of leaf gall inducers formed another cluster as *D. sueyenae* and the '*D. ornithocephala—D. stenocalia*' group.

DISCUSSION

An evaluation of the varied morphologies of galls induced by different species of *Daphnephila* on *Machilus thunbergii*, viewed against the morphometrics, nature of epidermis, numbers of layers of reserve- and active-nutritive tissue, the fungal species associated with the gall, and total-energy content confirmed that the assayed galls are extended phenotypes of *Daphnephila*.

The larvae of most gall-inducing Cecidomyiidae have a striking ability to manipulate plant tissues morphologically and physiologically, thus inducing galls to their advantage (Mani, 1964; Price et al., 1987; Rohfritsch, 2010). The high specificity trait of the Cecidomyiidae to their respective host plants and thus inducing galls of specific shapes as a surrogate of gall-inducing Cecidomyiidae led to the development of 'one shape on one host–one insect species' concept (Sýlven, 1979; Fernandes and Price, 1988; Price et al., 1998; Carneiro et al., 2009). However, a few exceptions do occur: for example, the parapatric populations of *Pseudasphondylia neolitseae* (Cecidomyiidae: Asphondyliini) on *Neolitsea sericea* (Lauraceae) induce two distinctly varied galls (Mishima and Yukawa, 2007) and two sympatric populations of *Hartigiola* (Cecidomyiidae: Ologitophini) on *Fagus crenata* (Fagaceae) induce apparently similar galls, one along the upper surfaces of leaves and the other on the lower surfaces, with sexual isolation manifesting on the same host (Mishima et al., 2014). Taiwanese *Daphnephila* induce varied types of galls, further to the five studied in this paper. Among those, some induce galls of similar morphologies on other species of *Machilus*. Since these gall-inducing *Daphnephila* are not yet named, whether they may challenge the 'one shape on one host–one species' concept remains unverified. At least the five galls induced by *D. truncicola, D. taiwanensis*, *D. sueyenae*, *D. stenocalia*, and *D. ornithocephala* on *M. thunbergii* lend support to the 'one shape on one host–one species' concept.

Galls induced by *Daphnephila* on Taiwanese *Machilus* demonstrate a common basic design, although variations in their external morphologies and in the numbers of cell layers constituting galls manifest. In our study, the fleshy galls induced by *D. taiwanensis* and *D. sueyenae* include more numbers of reserve and active nutritive cell layers, lower density of galls per leaf, and greater-water content than the slim galls induced by *D. truncicola,* and *D. stenocalia*. Since galls supply nutrients to the gall-inducing insect (Bronner, 1992; Abrahamson et al., 1998; Diamond et al., 2008; Raman, 2011), variations in the differentiation of gall tissues can affect the availability and supply of nutrients. As these galls are induced on the same host species and occasionally on the same host organ (viz., leaf), they indicate that gall shape, gall structure, and nutritive material in the galls are determined by the insects, reinforcing the classical experiments of Rohfrtitsch (1971).

In general, the chlorophyll content in galls on *Machilus* induced by *Daphnephila* has been shown to be low; chloroplasts in these galls also lack the photosynthetic pigment–protein complexes (Yang et al., 2003). Galls induced by *Bruggmanniella* sp. (Diptera: Cecidomyiidae) on *Litsea acuminata* (Lauraceae) have been shown to be devoid of stomata on their epidermises (Yang et al., 2008). The absence of stomata on the surfaces of the galls of *Daphnephila* on *Machilus* in Taiwan is critical in making us to suggest that no gas exchange supporting photosynthesis occurs thus affecting normal plant metabolism to some extent. *Daphnephila* is a monophyletic group and *D. truncicola*, which induces 'red' galls on stems, is shown to be the primitive species in this group (Tokuda et al., 2008). *Daphnephila taiwanensis*, which induces red, green and color-changing galls is shown to be in the ancestral clade among leaf gall-inducing taxa. The red gall-inducing *D. ornithocephala* and the two green gall-inducing Cecidomyiidae *D. sueyenae* and *D. stenocalia* sit in the derived unresolved clade of *Daphnephila* along with Japanese species, which induce green galls. Species of *Daphnephila* associated with *M. thunbergii* from Taiwan form a paraphyletic group, occurring at the basal position. This suggests that these species have speciated on *M. thunbergii* in Taiwan, and later in time shifted to *M. japonica* (Tokuda et al., 2008). Analysis of galls suggests that red galls of *D. taiwanensis* include greater anthocyanin content and carotenoid:chlorophyll ratio than the green galls of *D. taiwanensis* and *D. sueyenae* (Huang et al., 2011). Production of greater anthocyanin content could be a compensating mechanism to reduce damages associated with photons and reactive-oxygen species.

Galls function as a sink, mobilizing energy from normal plant organs (Janckiewicz et al., 1970; Kirst and Rapp, 1974; Larson and Whitham, 1991; Florentine et al., 2005; Raman et al., 2006). Energy content within a gall is affected by the abilities of gall-inducing insects so as to utilize nutrients from their host plants. As suggested by phylogenetic analysis (Tokuda et al., 2008), the stem gall inducer *D. truncicola* occurs at a basal position compared with the other four leaf-gall inducing *Daphnephila* species. Shifts in plant- organ use from stem to leaf represent adaptive radiation, which could be possibly due to greater energy availability to the larvae that live on the leaves of *M. thunbergii*. In the context of *Mangifera indica* (Anacardiaceae)-associated gall midges, only *Procontarinia mangiferae* lives both on stems and leaves of *M. indica*, indicating a critical step in *Procontarinia* speciation, through reproductive isolation involving phenological separation (Raman et al., 2009). Similar host-organ shifts are known on species of *Rhopalomyia* (Diptera: Cecidomyiidae) living on species of *Artemesia* (Asteraceae) and species of *Rabdophaga* (Diptera: Cecidomyiidae) living on species of *Salix* (Salicaceae) when assembled related data from various literatures (Nijveldt and Yukawa, 1982; Gagné, 1989; Yukawa et al., 2005; Russo, 2007; Skuhravá and Skuhravý, 2007; Gagné and Jaschhof, 2014). Such inter-organ shifts suggest effective utilization of the host plant and consequent speciation in the gall-inducing Cecidomyiidae. Occasionally *D. taiwanensis* occurs on petiole, although it typically induces galls on the leaf (Pan and Yang, unpublished observations). As this species is situated in the basal lineage among the leaf-gall inducing species of *Daphnephila*, along with the stem gall-inducing *D. truncicola* being ancestral to *Daphnephila*, *D. taiwanensis* possibly represents a transition phase of organ use from stem to leaf. Using varied plant organs on the same host as five gall-inducing Cecidomyiidae on the stems or leaves of *M. thunbergii* provides further evidences to verify the phylogenetic trend in the use of plant organs. *Machilus thunbergii* in Taiwan is pivotal for the radiation of the five species of *Daphnephila* by providing opportunities for these monophyletic inducers in selecting host organs via shifts.

The *D. truncicola*-induced galls on stems of *M. thunbergii* include the lowest energy content, despite having largest sizes in both gall and larva. The most basal species among leaf-gall inducers, *D. taiwanensis*, occurs at a transitory level, since their energy contents are the lowest among leaf galls, but greater than the galls induced by *D. truncicola*. Galls induced by *D. stenocalia* and *D. sueyenae* include higher energy contents than that in *D. taiwanensis*. The leaf galls induced by *D. ornithocephala* appear exceptional, with low levels of energy contents, lower density per leaf, and high water content, compared with the slim gall induced by *D. stenocalia*. It suggests that the selection forces may operate toward smaller sizes for both galls and insects and increasing nutrient efficiency with an optimal relationship. The *D. ornithocephala* which have middle body size in Taiwanese group speciated at interglacial period and the two smallest body size species, *D. sueyenae* and *D. stenocalia*, appear to have speciated at two different glacial period during the Pleistocene (Chiang and Yang, unpublished data). Our field surveys show that galls of *D. ornithocephala* are relatively rare as compared with other *Daphnephila* leaf-gall inducing species, and they occur often on stressed small trees in warmer places. These galls often cluster together in large numbers. When galls cluster together, the inhabiting larvae could face the intraspecific and interspecific competition (Larson and Whitham, 1997). Different strategies of nutrient utilization may be adopted by *D. ornithocephala* compared to its allies on *M. thunbergii* in Taiwan.

This paper reports the fungi from within galls of *Daphnephila* induced on *M. thunbergii*. Since the larvae of the Asphondyliini use a specific fungal species to activate plant cells and to extract nourishment from the host, fungal access to the vascular tissues of the host plant is the key for

gall growth and shape (Rohfritsch, 2008). However, in this study, we demonstrate the random pattern noted among the fungi in the galls. However, because *Botryosphaeria dothidea* was consistently isolated from the galls on the Taiwanese populations of *M. thunbergii* induced by species of *Daphnephila*, we infer that *B. dothidea* is the primary associate and possibly a symbiont. Earlier studies (Bissett and Borkent, 1988; Adair et al., 2009; Rohfritsch, 2008; Kobune et al., 2012) indicating that the larvae of Asphondyliini depended on *B. dothidea* for their development reinforce our inference. The different fungal taxa isolated from mature galls induced by the same *Daphnephila* populations suggest that these Cecidomyiidae may use different species of secondary fungal associates possibly to enhance their nutrition. The need to use multiple fungal associations may have resulted from warm, humid habitats, where the gall-inducing Cecidomyiidae are better adapted to collect spores of diverse fungi including saprophytic and endophytic fungi from the environment. The combination of specific and/or nonspecific multiple fungi appear in the phylogenetic basal clade of the subtribe Asphondyliina (Tokuda and Yukawa, 2007), while the evolved Cecidomyiidae occur associated with specific fungi. *Daphnephila* is situated in the most basal phylogenetic clades of subtribe Asphondyliina and is the singular genus with a soft ovipositor terminal (Tokuda and Yukawa, 2007). Whether the soft tip of the ovipositor plays a role with the multiple fungal association needs to be verified and confirmed.

We conclude that the gall shape and structure, in addition to multiple coexisting fungal combinations in galls, are influenced by species of *Daphnephila* living on *M. thunbergii* in Taiwan. The fleshy and slim galls of *Daphnephila* include varying numbers of cell layers. Gall energy contents are determined by structures of reserve and nutritive cell layers and the fungi associated with the gall midge species. Our results verify that shapes, structure, nutritive energy, and multiple coexisting fungal combinations of galls represent extended phenotypes of the gall-inducing *Daphnephila* on *M. thunbergii* in Taiwan.

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