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Application of cecidomyiid galls to the systematics of the genus *Machilus* (Lauraceae) in Taiwan

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Abstract. The species of the genus *Machilus* (Lauraceae) in Taiwan sustain diverse cecidomyiid galls induced by the insects of the genus *Daphnephila* (Cecidomyiidae). This work examines the feasibility of applying cecidomyiid galls to the systematics of the genus *Machilus*. Amplified fragment length polymorphism (AFLP) was used to analyze the 38 gall-bearing trees of four *Machilus* taxa including *Machilus kusanoi*, *M. thunbergii*, *M. zuihoensis* var. *zuihoensis*, and *M. zuihoensis* var. *mushaensis*. The UPGMA cluster analysis of the AFLP data revealed three distinct clusters, including *M. kusanoi*, *M. thunbergii*, and *M. zuihoensis* variety complex. *Machilus zuihoensis* var. *zuihoensis* and *M. zuihoensis* var. *mushaensis* were indistinguishable from the three primer combinations. These two varieties could be considered the same taxon. PCR and DNA sequencing methods were used to analyze the nucleotide sequences of the mitochondrial 16S rDNA gene of the twenty gall midges from three types of galls from four *Machilus* taxa. The phylogenetic tree from the partial 16S rDNA sequence by UPGMA method of proportion distance revealed that the gall midges can be divided into three groups according to gall types. The phylogenetic tree cannot separate the two varieties of *M. zuihoensis* within each group. *Machilus zuihoensis* var. *zuihoensis* and *M. zuihoensis* var. *mushaensis* cannot be distinguished according to the AFLP or DNA sequencing methods, and they are more closely related to *M. thunbergii* than to *M. kusanoi*. The systematic relationships among the *Machilus* from the data of host plants are congruent with the data from the gall inducers. Results in this study imply that the gall inducers of genus *Daphnephila* provide information for resolving the plant systematic relationships based on molecular techniques.

Keywords: AFLP; Cecidomyiidae; DNA sequence; Insect gall; *Machilus*; Systematics.

Introduction

The genus *Machilus* of Lauraceae is distributed in the tropical and subtropical areas of Asia (Liu et al., 1994). There are six taxa of *Machilus* in Taiwan: *Machilus kusanoi*, *M. obovatifolia*, *M. zuihoensis* var. *zuihoensis*, *M. zuihoensis* var. *mushaensis*, *M. thunbergii* and *M. japonica*. All except *M. thunbergii* and *M. japonica* are endemic species in Taiwan. Currently, plant taxonomists differ in their systematic arrangement of the *Machilus* taxa in Taiwan.

A gall is an abnormal growth on some part of a plant as a result of the activity of another organism (virus, nematode, arthropod etc.) and the gall inducer uses this structure as a shelter and nutrition source (Csóka, 1997). The genus *Machilus* plants in Taiwan have a high diversity of cecidomyiid galls induced by the insects of the genus *Daphnephila* (Cecidomyiidae). Harris (1994) demonstrated that the family Cecidomyiidae is one of the major groups of gall inducers and that most gall midges are highly host specific. The galls of phytophagous arthropods are traits associated with plants and may be useful for separating plant taxa (Floate et al., 1996). Abrahamson et al. (1998) indicated that cynipid occurrences offer helpful information concerning some aspects of oak systematics. Our work examines the feasibility of applying cecidomyiid galls to the systematics of the genus *Machilus* (Lauraceae) in Taiwan. Amplified fragment length polymorphism (AFLP) was used to analyze the phylogeny of *Machilus* taxa. PCR and DNA sequencing methods were used to examine the phylogeny of gall inducers gathered from galled plants.

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Materials and Methods

Gall-Bearing Plants

Plant materials. Thirty eight gall-bearing trees of four *Machilus* taxa including *Machilus kusanoi* (code K), *M. thunbergii* (T), *M. zuihoensis* var. *zuihoensis* (Z) and *M. zuihoensis* var. *mushaensis* (M) were sampled throughout Taiwan (Figure 1). Leaf samples from each plant were packed in a paper bag and stored in a box of silica gel desiccant. The dried leaves were used for DNA extraction. Only four *Machilus* taxa were analyzed in the preliminary study because there were not enough *M. obovatifolia* samples that are only distributed on the Hengchun Peninsula, and *M. japonica* was difficult to identify.

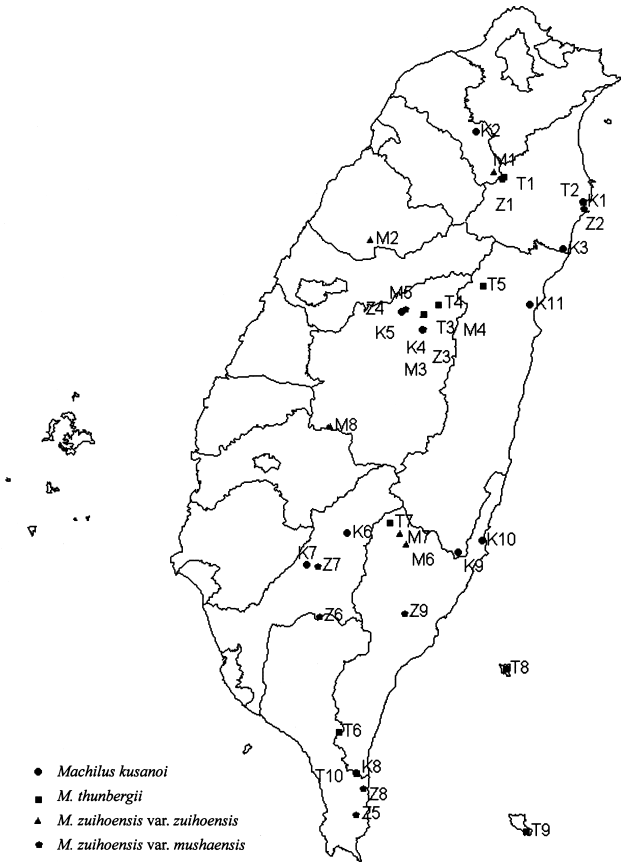

Figure 1. The *Machilus* collection sites in Taiwan.

DNA extraction. The leaves of the *Machilus* contain a large amount of polysaccharide that inhibits DNA extraction. DNA was extracted from dried leaf material according to the Kobayashi et al. method (1998) so that polysaccharides and polyphenolics could be removed from the DNA extraction protocol and high quality DNA could be extracted.

AFLP analysis. The AFLP technique, as reported by Vos et al. (1995), consists of three major steps: (1) restriction endonuclease digestion of the DNA and ligation of the adapters, (2) amplification of the restricted fragments, (3) gel analysis of the amplified fragments. The AFLP procedure was performed using the AFLP analysis system I (Life Technologies, Inc.) according to the manufacturer's instructions. The total genomic DNA was restricted with *Eco* RI and *Mse* I. The DNA fragments were ligated to *Eco* RI and *Mse* I adapters provided in the kit. Selective PCR was performed using three primer pairs: *Eco* RI+AAC and *Mse* I+CAA, *Eco* RI+ACC and *Mse* I+CTC, and *Eco* RI+ACG and *Mse* I+CAA.

Data analysis. The presence/absence of each scorable fragment was recorded in a binary data matrix. Data from the three primer combinations were combined, and the pairwise similarities between samples were calculated using a simple matching coefficient. The resultant similarity matrix was input into both a UPGMA cluster analysis and a principal coordinate analysis using NTSYS-PC, Version 2.0 (Rohlf, 1993).

Gall-Inducing Insects

Insect materials. Twenty specimens were gathered from three types of midge galls, including urn-shaped, mouse-like, and coniform galls obtained from four *Machilus* taxa

Table 1. The specimen data of gall-induced insects.

Specimen code	Kinds of gall	Host plant
UK1	Urn-shaped	*Machilus kusanoi*
UK2	Urn-shaped	*M. kusanoi*
UK3	Urn-shaped	*M. kusanoi*
UK4	Urn-shaped	*M. kusanoi*
UM1	Urn-shaped	*M. zuihoensis* var. *mushaensis*
UM2	Urn-shaped	*M. zuihoensis* var. *mushaensis*
UM3	Urn-shaped	*M. zuihoensis* var. *mushaensis*
UM4	Urn-shaped	*M. zuihoensis* var. *mushaensis*
UZ1	Urn-shaped	*M. zuihoensis* var. *zuihoensis*
UZ2	Urn-shaped	*M. zuihoensis* var. *zuihoensis*
MK1	Mouse-like	*M. kusanoi*
MK2	Mouse-like	*M. kusanoi*
MM1	Mouse-like	*M. zuihoensis* var. *mushaensis*
MM2	Mouse-like	*M. zuihoensis* var. *mushaensis*
MT1	Mouse-like	*M. thunbergii*
MZ1	Mouse-like	*M. zuihoensis* var. *zuihoensis*
CK1	Coniform	*M. kusanoi*
CM1	Coniform	*M. zuihoensis* var. *mushaensis*
CM2	Coniform	*M. zuihoensis* var. *mushaensis*
CM3	Coniform	*M. zuihoensis* var. *mushaensis*
CZ1	Coniform	*M. zuihoensis* var. *zuihoensis*
CZ2	Coniform	*M. zuihoensis* var. *zuihoensis*

(Table 1). The specimens were preserved in 70% alcohol at room temperature.

DNA extraction. The entire insect body was homogenized by a glass homogenizer in 500 µl digestion buffer that contained 100 mM Tris-Cl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 0.5% SDS, 50 mM dithiothreitol, and 0.5 mg/l proteinase K. The mixture was shaken horizontally (180 rpm) at 50°C overnight. The DNA template was generated using a phenol-chloroform extraction of total nucleic acids (Yeh and Yang, 1997). The extracted crude DNA was dissolved in 50 µl distilled H_2O.

PCR and DNA sequencing. A polymerase chain reaction (PCR) was employed to amplify part of the 3´ end sequence of the mitochondrial 16S rDNA gene. The primers used to amplify the region were 5´-GCCTGTTTA TCAAAAACAT-3´ and 5´-CCGGTCTGAACTCAGATCA-3´. PCR amplification was performed in a 100 µl reaction mix that contained 100 mM Tris-Cl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Trutib-X100, 2U SuperTag polymerase (HT Biotechnology, LTD), 0.2 mM of each dNTP, 20 pmoles of each primer, and 2 µl DNA template. A Perkin-Elmer 9600 thermal cycler was employed with the following temperature profile: 95°C for 2 min; 39 cycles of 95°C for 40 s, 48°C for 1 min, and 72°C for 30 s; 72°C for 10 min; and 4°C at the end. The target DNA was recovered from the gel by a Bio101 kit after electrophoresis, and the DNA products were sequenced directly using the AmpliCycle Sequencing kit (Perkin Elmer) for 29 cycles with the following temperature profiles: 95°C for 30 s, 55°C for 30 s, and 72°C for 20 s.

Data analysis. The partial 16S rDNA gene sequences were aligned using the GCG (Genetic Computer Group, Version 7.0) Pileup program (Devereux et al., 1991) and then visually checked. Phylogentic analysis of the aligned nucleotide sequences was performed by the UPGMA method of proportion distance using the MEGA program (Kumar et al., 1993). A bootstrap analysis was performed for 1000 replications in the clustering method.

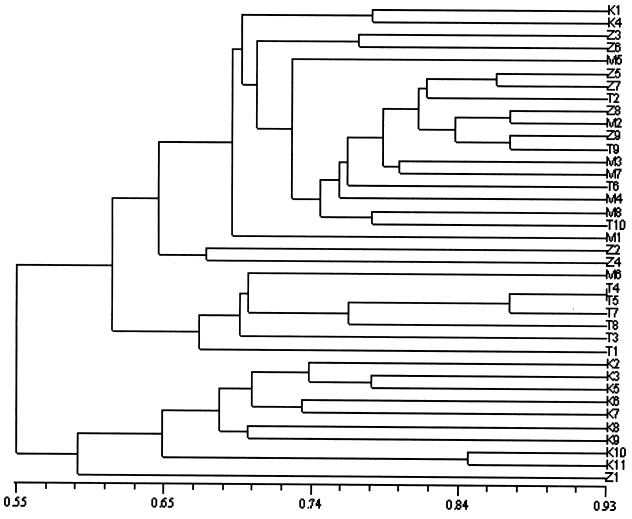

Figure 2. The dendrogram of the gall-bearing plants based on polymorphic AFLP bands.

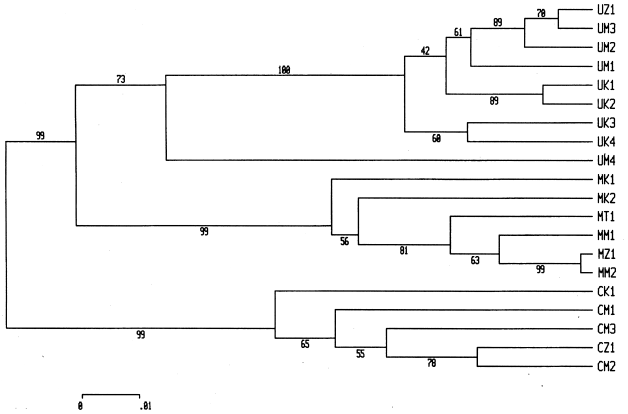

Figure 3. The phylogenetic tree inferred from the partial 16S rDNA sequence by the UPGMA method of proportion distance. The values (percentages) on the branches were the result of 1000 bootstrap replications.

Results

The three primer combinations employed for the AFLP of gall-bearing plants revealed 114 polymorphic bands. The number of polymorphic bands differed depending upon the primer combinations. The UPGMA cluster analysis revealed three distinct groups (Figure 2) and the cophenetic correlation coefficient was 0.858. The three distinct groups were categorized into *Machilus kusanoi*, *M. thunbergii*, and the *M. zuihoensis* variety complex. *Machilus zuihoensis* var. *zuihoensis* and *M. zuihoensis* var. *mushaensis* were indistinguishable, and they appeared to be the same taxon. Taxonomists disagree about the classification of the genus *Machilus* in Taiwan. The interference with the clustering may result from the difficulty of specimen identification.

GenBank accession numbers of the partial 16S rDNA gene sequence data of all twenty individual gall inducers are AF334186—AF334205. The average nucleotide base compositions of guanine, adenine, thymine and cytosine were 10.1%, 40.1%, 44.4% and 5.4%, respectively. The phylogenetic tree constructed by the UPGMA method using proportion distance could be divided into three groups according to the gall types: urn-shaped, mouse-like, and coniform galls (Figure 3). The bootstrap and distance analysis is illustrated in the tree (Figure 3). There is over 90% support for monophyly of each gall type gathered from the various *Machilus* taxa. The phylogenetic tree of each group cannot separate the two varieties of *M. zuihoensis*. The synthetic systematic relationships of the four *Machilus* taxa from DNA data of gall inducers imply that the two varieties of *M. zuihoensis* could be considered the same taxon, and they are more closely related to *M. thunbergii* than to *M. kusanoi*.

Discussion

Knowledge of the systematics of genus *Machilus* in Taiwan is limited because the morphological characteris-

tics are often confused. Two troublesome *Machilus* taxa are *M. zuihoensis* var. *mushaensis* and *M. japonica*. The two varieties of *Machilus zuihoensis* were considered two species by Yang et al. (1999), whereas Liu et al. (1994) categorized them as two varieties. *Machilus japonica* was considered as *M. pseudolongifolia* by Yang et al. (1999) and was often confused with *M. kusanoi*. Taxonomists differ in the systematics of the *Machilus* taxa using morphological characteristics such as wood anatomy (Chang, 1994), pollen morphology (Huang, 1972), ultra-structure of leaf epidermis (Ou, 1989), and biochemistry (Ou, 1975; Kuo, 1985) alone. We used the molecular technique AFLP to examine the systematics and found that *Machilus zuihoensis* var. *zuihoensis* and *M. zuihoensis* var. *mushaensis* may be the same taxa and they are more closely related to *M. thunbergii* than to *M. kusanoi*. Analysis results imply a coevolution between the *Machilus* plants and the *Daphnephila* gall inducers.

The Cecidomyiidae probably began plant feeding during the flowering plant radiation period in the late Cretaceous (Roskam, 1992). Cecidomyiidae presumably were pre-adapted for plant feeding and gall-inducing in an earlier period of angiosperm radiation (Roskam, 1992). The Cecidomyiidae family of insects is a major group of gall inducers, and most gall midges are highly host specific (Harris, 1994). The genus *Daphnephila* of Cecidomyiidae has only been recorded in India (Gagné, 1973) and Japan (Yukawa and Masuda, 1996). The gall inducer specimens gathered in Taiwan are difficult to identify into species level.

Phytophagous insects are a natural bioassay that may be used to segregate closely related plant taxa (Floate and Whitham, 1995). The distributions of gall inducers may serve to distinguish hybrid plants (Aguilar and Boecklen, 1992; Fritz et al., 1994; Floate and Whitham, 1995) and even intrapopulational categories of plant genotypes (Floate et al., 1996). The systematic relationships of four *Machilus* taxa gathered from the data of gall inducers herein are congruent with the data gathered from gall-bearing plants. These results suggest that gall midges *Daphnephila* can distinguish the characteristics among *Machilus* taxa and can help resolve plant systematic problems. These cecidomyiid galls on *Machilus* may have already taken a long time to adapt to the host plants in this region. Future studies will collect more specimens of gall-bearing plants and gall-inducing insects to clarify the intact systematic relationships of genus *Machilus* and genus *Daphnephila*.

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癭蚋蟲癭在臺灣楨楠屬植物系統分類學之應用

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臺灣楨楠屬（*Machilus*）植物有多樣的癭蚋蟲癭（cecidomyiid gall），造癭者為癭蚋科的 *Daphnephila* 屬。本研究探討癭蚋蟲癭在臺灣楨楠屬植物系統分類學之可行性。在產癭植物方面，使用 AFLP 方法分析大葉楠（*Machilus kusanoi*）、豬腳楠（*M. thunbergii*）、香楠（*M. zuihoensis* var. *zuihoensis*）及霧社楨楠（*M. zuihoensis* var. *mushaensis*）的 38 個樣本，以 UPGMA 方法進行歸群分析，可明顯分成 3 群。AFLP 的 3 個引子組的結果無法區分香楠及霧社楨楠，兩者可歸於同一分類群。在造癭昆蟲方面，以 PCR 及 DNA 定序分析採自 3 型蟲癭的 20 個樣本的粒線體 16S rDNA 序列，以 proportion distance 依 UPGMA 歸群方法所得的樹形圖顯示所分析的造癭昆蟲可依蟲癭類型分成 3 群，每一群皆無法區分香楠與霧社楨楠。本研究發現，根據 DNA 定序與 AFLP 的方法，皆無法區分香楠與霧社楨楠，且此兩者與豬腳楠的親源關係較大葉楠親近。楨楠屬產癭植物與 *Daphnephila* 屬造癭昆蟲所得的楨楠屬植物系統關係符合，*Daphnephila* 屬造癭昆蟲可協助釐清楨楠屬植物之系統關係。

關鍵詞：AFLP；癭蚋科；DNA序列；蟲癭；楨楠屬；系統學。