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.

**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.
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\textsubscript{RI} and Mse\textsubscript{I}. The DNA fragments were ligated to Eco\textsubscript{RI} and Mse\textsubscript{I} adapters provided in the kit. Selective PCR was performed using three primer pairs: Eco\textsubscript{RI}+AAC and Mse\textsubscript{I}+CAA, Eco\textsubscript{RI}+ACC and Mse\textsubscript{I}+CTC, and Eco\textsubscript{RI}+ACG and Mse\textsubscript{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.

<table>
<thead>
<tr>
<th>Specimen code</th>
<th>Kinds of gall</th>
<th>Host plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK1</td>
<td>Urn-shaped</td>
<td><em>Machilus kusanoi</em></td>
</tr>
<tr>
<td>UK2</td>
<td>Urn-shaped</td>
<td><em>M. kusanoi</em></td>
</tr>
<tr>
<td>UK3</td>
<td>Urn-shaped</td>
<td><em>M. kusanoi</em></td>
</tr>
<tr>
<td>UK4</td>
<td>Urn-shaped</td>
<td><em>M. kusanoi</em></td>
</tr>
<tr>
<td>UM1</td>
<td>Urn-shaped</td>
<td><em>M. zuihoensis</em> var. mushaensis</td>
</tr>
<tr>
<td>UM2</td>
<td>Urn-shaped</td>
<td><em>M. zuihoensis</em> var. mushaensis</td>
</tr>
<tr>
<td>UM3</td>
<td>Urn-shaped</td>
<td><em>M. zuihoensis</em> var. mushaensis</td>
</tr>
<tr>
<td>UM4</td>
<td>Urn-shaped</td>
<td><em>M. zuihoensis</em> var. mushaensis</td>
</tr>
<tr>
<td>UZ1</td>
<td>Urn-shaped</td>
<td><em>M. zuihoensis</em> var. zuihoensis</td>
</tr>
<tr>
<td>UZ2</td>
<td>Urn-shaped</td>
<td><em>M. zuihoensis</em> var. zuihoensis</td>
</tr>
<tr>
<td>MK1</td>
<td>Mouse-like</td>
<td><em>M. kusanoi</em></td>
</tr>
<tr>
<td>MK2</td>
<td>Mouse-like</td>
<td><em>M. kusanoi</em></td>
</tr>
<tr>
<td>MM1</td>
<td>Mouse-like</td>
<td><em>M. zuihoensis</em> var. mushaensis</td>
</tr>
<tr>
<td>MM2</td>
<td>Mouse-like</td>
<td><em>M. zuihoensis</em> var. mushaensis</td>
</tr>
<tr>
<td>MT1</td>
<td>Mouse-like</td>
<td><em>M. thunbergii</em></td>
</tr>
<tr>
<td>MZ1</td>
<td>Mouse-like</td>
<td><em>M. zuihoensis</em> var. zuihoensis</td>
</tr>
<tr>
<td>CK1</td>
<td>Coniform</td>
<td><em>M. kusanoi</em></td>
</tr>
<tr>
<td>CM1</td>
<td>Coniform</td>
<td><em>M. zuihoensis</em> var. mushaensis</td>
</tr>
<tr>
<td>CM2</td>
<td>Coniform</td>
<td><em>M. zuihoensis</em> var. mushaensis</td>
</tr>
<tr>
<td>CM3</td>
<td>Coniform</td>
<td><em>M. zuihoensis</em> var. mushaensis</td>
</tr>
<tr>
<td>CZ1</td>
<td>Coniform</td>
<td><em>M. zuihoensis</em> var. zuihoensis</td>
</tr>
<tr>
<td>CZ2</td>
<td>Coniform</td>
<td><em>M. zuihoensis</em> var. zuihoensis</td>
</tr>
</tbody>
</table>
(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 protease 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 H2O.

**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 TCAAAACAT-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 MgCl2, 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. Phylogenetic 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.

**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. mushuaensis 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. mushuaensis 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 (Flatoe and Whitham, 1995). The distributions of gall inducers may serve to distinguish hybrid plants (Aguilar and Boecklen, 1992; Fritz et al., 1994; Flatoe and Whitham, 1995) and even intrapopulational categories of plant genotypes (Flatoe 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 gallers 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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癢蚋蟲瘿在臺灣檜楠屬植物系統分類學之應用

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臺灣檜楠屬（Machilus）植物有多種的癢蚋蟲瘿（cecidomyiid gall），治療者為癢蚋科的Daphnephila屬。本研究探討癢蚋蟲瘿在臺灣檜楠屬植物系統分類學之可行性。在產殼植物方面，使用AFLP方法分析大葉楠（Machilus kusanoi）、狹葉楠（M. thunbergii）、香楠（M. zuihoensis var. zuihoensis）及霧社檜楠（M. zuihoensis var. mushaensis）的 38 個樣本，以UPGMA方法進行歸類分析，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楠楠，兩者可歸於同一分類群。在造殼昆蟲方面，以PCR及DNA定序分析採自 3 型蟲瘿的 20 個樣本的粒線體 16S rDNA 序列，以 proportion distance 依 UPGMA 歸類方法所得的樹形圖顯示所分析的造殼昆蟲可依蟲瘿類型分成 3 群，每一群皆無法區分香楠與霧社楠楠。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楠楠，且此兩者與猩葉楠的親源關係較大葉楠親近。檜楠屬產殼植物與 Daphnephila 屬造殼昆蟲所得的檜楠屬植物系統關係符合，Daphnephila 屬造殼昆蟲可協助釐清檜楠屬植物之系統關係。

關鍵詞：AFLP；癢蚋科；DNA序列；蟲瘿；檜楠屬；系統學。