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Full Length Research Paper

Cladistic relationships within the genus *Cinnamomum* (Lauraceae) in Taiwan based on analysis of leaf morphology and inter-simple sequence repeat (ISSR) and internal transcribed spacer (ITS) molecular markers

Kuen-Yih Ho* and Tsz-Yao Hung

Department of Forestry and Natural Resources, National Chiayi University, 300 Xuefu Road, Luliao Vil., Chiayi 60004, Taiwan.

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We examined leaf morphological characteristics, ISSR (inter-simple sequence repeat) genetic polymorphisms and ITS (rDNA internal transcribed spacer) molecular markers in 12 endemic species of *Cinnamomum* in Taiwan to determine their cladistic relationships. The leaf morphology and ISSR data support the division of the genus into sections *Camphora* and *Cinnamomum*. The genetic relationship between *Cinnamomum camphora* and *Cinnamomum micranthum* is very close; both species share a specific 11 bp deletion in their ITS sequences. A close relationship between *Cinnamomum insularimontanum* and *Cinnamomum macrostemon was* supported by leaf morphology, ISSR and ITS data and the ITS analysis indicates that *Cinnamomum subavenium* is closely related to these two species. The ITS analysis also indicates that *Cinnamomum japonicum*, *Cinnamomum austrosinense* and *Cinnamomum reticulatum* are closely related. Leaf morphology and ISSRs also support the kinship between *C. japonicum* and *C. austrosinense*. The ITS data support a close cluster consisting of *C. osmophloeum*, *C. camphora* and *C. micranthum*, suggesting that *Cinnamomum osmophloeum* might be a key species in the evolutionary transition from section *Camphora* to section *Cinnamomum*. Our results demonstrate that ISSR and ITS markers can clearly identify the 12 endemic *Cinnamomum* species in Taiwan.

Key words: *Cinnamomum*, morphology, taxonomy, ISSR (inter-simple sequence repeat), ITS (internal transcribed spacer), phylogeny.

INTRODUCTION

The genus *Cinnamomum* of the family Lauraceae contains about 250 species worldwide, mostly distributed in tropical and subtropical Asia. The 12 endemic species of the genus in Taiwan are evergreen trees or shrubs distinguished by their prominently trinervate leaves and drupaceous fruits set on top of thick, cuplike receptacles (Chang, 1976). Since ancient times, cinnamon bark from members of the genus has been a mainstay of culinary and medicinal applications. The wood of most trees in the genus is also treasured as a good material for furniture making, carving and structural uses. Tree species in the genus often possess pleasing growth forms that grace urban streets. Commercial camphor, which is extracted from the wood of *Cinnamomum camphora*, is an important product that was once highly valuable to the economy of Taiwan. Thus, trees of this genus are economically important forest resources.

*Corresponding author. E-mail: kyho@mail.ncyu.edu.tw. Tel: +886-5-2717463.

Abbreviations: ISSR, Inter-simple sequence repeat; **ITS,** internal transcribed spacer; **NJ,** neighbor joining; **MP,** maximum parsimony; **PCR,** polymerase chain reaction; **ETS,** external transcribed spacer; **OUT,** operational taxonomic unit; **UPGMA,** unweighted pair group method with arithmetic mean; **CTAB,** cetyl trimethyl ammonium bromide; **DMSO,** dimethyl sulfoxide; **MCL,** maximum composite likelihood; **NCBI,** National Center for Biotechnological Information.

The genus *Cinnamomum* has a rather ancient origin and wide distribution, with distinguished ecological and economic significance. However, relatively few studies have examined its evolutionary systematics. Therefore, many taxonomic controversies remain. To date, studies of the genus in Taiwan have mostly concentrated on phenotypic evidence from external morphology or microscopic examination of the wood and leaves (Huang, 1984; Ou, 1989; Chang, 1995). The main justification for these modes of study was the lack of widely accepted systematic hypotheses. Many differing classification schemes were derived from different interpretations of the external morphological characteristics. Thus, the application of novel molecular biological evidence is needed to resolve the systematic controversy (Li and Li, 2004).

In recent years, molecular markers have been widely applied in plant systematics and have helped to resolve many ambiguous or subjective misclassifications. These techniques allow direct and accurate analysis of genetic variations that may not be morphologically apparent. The greatest advantage of molecular markers is that they directly reflect variation at the molecular level and thus, provide an objective view of molecular evolution. Thus, the characters to be analyzed are not influenced by the growth environment or growth stage of the plant. Furthermore, PCR applications require only a minute quantity of a specimen template for a comprehensive analysis. Thus, for the investigation of cladistic relationships between morphologically similar species, these techniques provide not only objective evidence but also convenient and suitable analytical tools. Ho (2006) has successfully used ISSR molecular markers to study the genetic variation and taxonomic status of *Cinnamomum osmophloeum*, *Cinnamomum macrostemon* and *Cinnamomum insularimontanum*. Li et al. (2006) and Li et al. (2007) have analyzed the ITS and ETS sequences of ribosomal genes to determine the systematic relationships of the related genera *Actinodaphne* and *Neolitsea*. Molecular markers are extensively applied in studies of species identification, genetic map construction, interspecific relationships, evolutionary systematics and biogeography (Lei et al., 2004; Kunjupillai and Tsou, 2008; Xiao et al., 2008; Chen et al., 2009).

In this study, we applied inter-simple sequence repeat (ISSR) markers and nuclear ribosomal DNA internal transcribed spacer (ITS) sequences in conjunction with leaf morphological characteristics (using ANOVA and phenetic clustering) to delineate the cladistic relationships among the 12 *Cinnamomum* species in Taiwan.

MATERIALS AND METHODS

Plant material was extensively collected from 16 natural population sites in Taiwan and Lanyu (Figure 1). For each species, 3 to 5 specimens were collected from different sites (Table 1). From each sampled tree, 10 mature, damage-free leaves were collected, cleaned and disinfected with 70% alcohol and preserved in a desiccating bag filled with silica gel.

Observations of leaf morphological characteristics

In the laboratory, the length, length-to-width ratio, apex angle, base angle and venation pattern of each leaf were measured. The numerical data were analyzed using the program NTSYS (Rohlf, 1993) to derive clusters (Eeuclidean distances) of similar specimens. The Euclidean distances among the clusters were calculated and used to generate a cladogram using the UPGMA algorithm.

ITS and ISSR molecular markers

A modification of the CTAB method described by Kobayashi et al. (1998) was followed to extract genomic DNA from the leaf samples.

ITS sequence analyses

The ITS primer sequences used in this study were obtained from the Biomedical Engineering Research Laboratories of the Industrial Technology Research Institute at Hsinchu, Taiwan (Chiou et al., 2007). PCR amplification of the ITS sequences entailed an initial denaturation cycle at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 50 s, annealing at 57°C for 50 s and extension at 72°C for 2 min and a final extension at 72°C for 10 min. The reaction mixture contained 5 µl of a 10x PCR buffer, 4 µl of 2.4 mM dNTPs, 3 µl of DNA template (10 ng/µl), 1.5 µl of 5% dimethyl sulfoxide (DMSO), 1 µl of each primer, 0.6 µl of 20 mM $MgCl_2$ and 0.4 µl of Taq DNA polymerase (1 U) in a total volume of 50 µl.

The PCR-amplified products were loaded on a horizontal electrophoresis system (IBI HR 2025) containing a 1.8% agarose gel. A 0.5x TBE buffer solution was added and an electrical charge of 100 V was applied to separate the bands. The agarose gels were then stained in a 0.5 µg ml^{-1} ethidium bromide solution. In a darkroom, the gels were observed and photographed under UV light (EZ lab Uni-photo) to document the bands.

The pGEM-T easy vector system (Promega) was utilized to clone individual copies of the ITS sequences. Cloned fragments were then sequenced by the Mission Biotech Company. The DNA sequences were initially aligned using the ClustalV algorithm within the program MegAlign (Dnastar, Lasergene) (Higgins and Sharp, 1989). Gaps in the sequences were manually coded as missing data. The resulting sequences were then analyzed using the MEGA v.4.0 software package (Tamura et al., 2007) to determine the frequencies and ratios of transitions and transversions. The model of nucleotide substitution was inferred using maximum composite likelihood (MCL) analysis in MEGA v.4.0, in which the combined variability of all sites is used to estimate the evolutionary distance between sequences without the need to consider the differences in nucleotide substitutions between various sequences. Phylogenetic trees were constructed using the neighbor-joining (NJ) (Saitou and Nei, 1987) and maximum parsimony (MP) methods (Fitch, 1971). Node support was determined from 1,000 bootstrap replicates to validate the NJ and MP consensus trees (Felsenstein, 1985).

ISSR marker analysis

The procedures followed to generate the ISSR data for the sampled trees were essentially the same as those followed to obtain the ITS sequences. Seven primers were selected from the University of British Columbia genetic marker library (UBC 801-900) (Table 2) and used to amplify ISSR fragments. The molecular results were recorded and analyzed using NTSYS. Using the simple matching

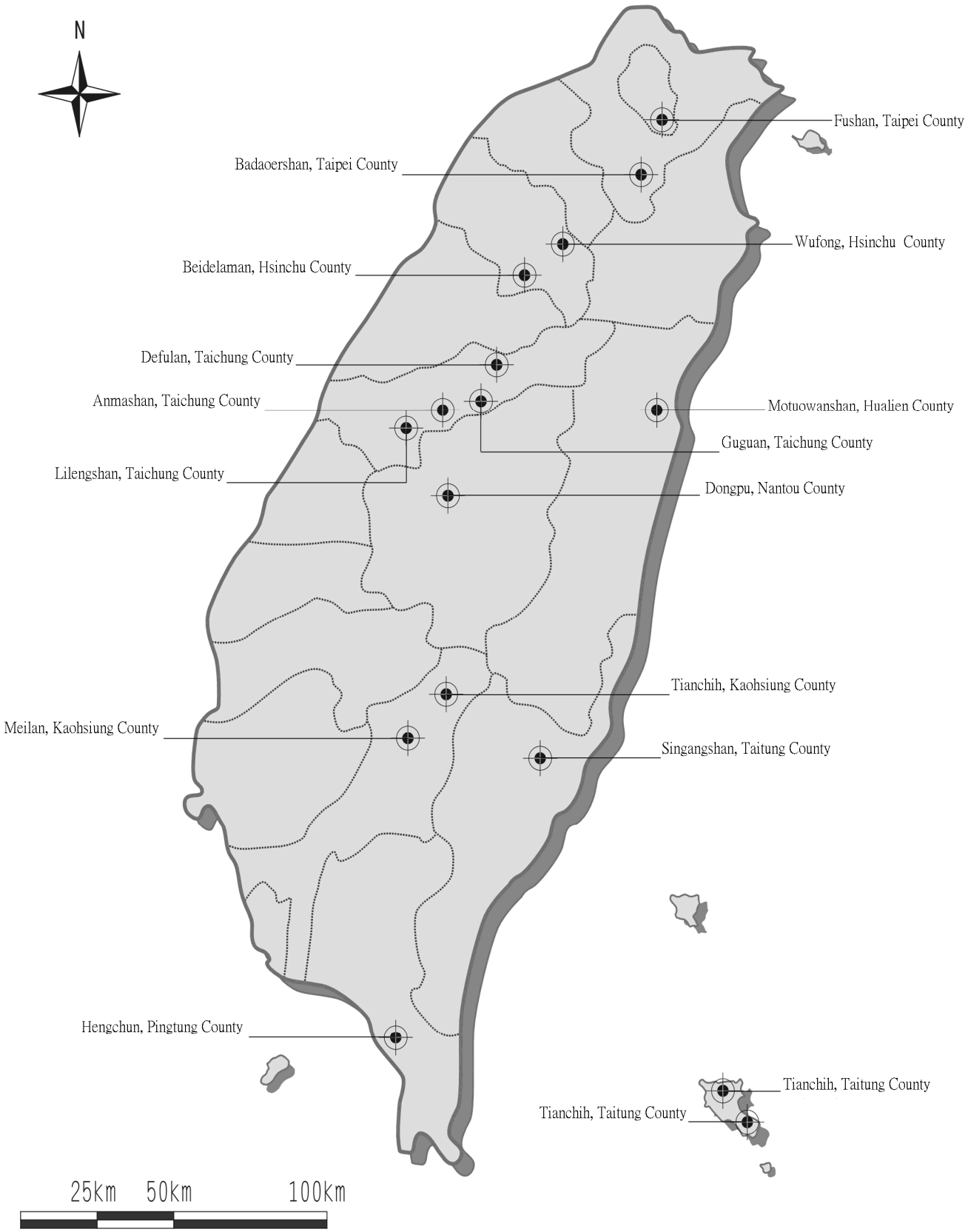

Figure 1. Taiwanese collection sites of *Cinnamomum* spp.

Table 1. Localities of *Cinnamomum* samples.

Taxon	Collected site	OTU number	DNA code
C. brevipedunculatum	Hengchun, Pingtung County	br-H1	C.br_H1
	Hengchun, Pingtung County	br-H2	C.br_H2
	Hengchun, Pingtung County	br-H3	C.br_H3
	Fushan, Taipei County	br-F1	
C. camphora	Fushan, Taipei County	ca-F1	C.ca_F1
	Fushan, Taipei County	ca-F2	
	Fushan, Taipei County	ca-F3	C.ca_F3
	Fushan, Taipei County	ca-F4	
	Fushan, Taipei County	ca-F5	C.ca_F5
C. insularimontanum	Dongpu, Nantou County	in-D1	C.in_D
	Dongpu, Nantou County	in-D4	C.in_D41
	Meilan, Kaohsiung County	in-M6	C.in_M6
	Meilan, Kaohsiung County	in-M8	
C. japonicum Tianchih	Taitung County	ja-T13	C.ja_T13
	Taitung County	ja-T14	C.ja_T14
	Taitung County	ja-T15	
	Siaotianchih, Taitung County	ja-S4	C.ja_S4
C. kotoense Hengchun	Pingtung County	ko-H1	
	Pingtung County	ko-H2	C.ko_H2
	Pingtung County	ko-H3	C.ko_H3
	Orchid Island, Taitung County	ko-L2	
	Orchid Island, Taitung County	ko-L3	C.ko_L3
C. macrostemon Wufong	Hsinchu County	ma-W1	C.ma_W1
	Hsinchu County	ma-W2	
	Singangshan, Taitung County	ma-S2	C.ma_S2
	Motuowanshan, Hualien County	ma-M1	C.ma_M1
C. micranthum	Fushan, Taipei County	mi-F1	C.mi_F1
	Fushan, Taipei County	mi-F2	C.mi_F2
	Fushan, Taipei County	mi-F3	C.mi_F3
	Fushan, Taipei County	mi-F4	
C. osmophloeum	Anmashan, Taichung County	os-A1	C.os_A1
	Lilengshan, Taichung County	os-L1	C.os_L1
	Lilengshan, Taichung County	os-L2	
	Defulan, Taichung County	os-D1	
	Defulan, Taichung County	os-D7	C.os_D7
C. philippinense	Fushan, Taipei County	ph-F1	
	Fushan, Taipei County	ph-F2	C.ph_F2
	Fushan, Taipei County	ph-F3	C.ph_F3
	Fushan, Taipei County	ph-F4	C.ph_F4
C. austro-sinense	Guguan, Taichung County	au-G1	
	Guguan, Taichung County	au-G2	
	Badaoershan, Taipei County	au-B2	C.au_B2
	Fushan, Taipei County	au-F3	C.au_F3

Table 1. Contiunes

	Fushan, Taipei County	au-F5	
	Tianchih, Kaohsiung County	au-T10	C.au_T10
	Badaoershan, Taipei County	su-B7	
	Badaoershan, Taipei County	su-B8	C.su_B8
C. subavenium	Guguan, Taichung County	su-G1	
	Guguan, Taichung County	su-G2	C.su_G2
	Beidelaman, Hsinchu County	su-D9	C.su_D9
	Hengchun, Pingtung County	re-H1	C.re_H1
C. reticulatum	Hengchun, Pingtung County	re-H2	C.re_H2
	Hengchun, Pingtung County	re-H5	C.re_H5

Table 2. Primer sequences and annealing temperature for ISSR analysis.

Primer number	Sequence (5' to 3')	Annealing temperature (°C)
UBC 823	TCT CTC TCT CTC TCT CC	54
UBC 825	ACA CAC ACA CAC ACA CT	54
UBC 827	ACA CAC ACA CAC ACA CG	56
UBC 842	GAG AGA GAG AGA GAG ACT G	56
UBC 855	ACA CAC ACA CAC ACA CCT T	53
UBC 856	ACA CAC ACA CAC ACA CTA	53
UBC 864	ATG ATG ATG ATG ATG ATG	52

mode, the similarity index between each pair of specimens was calculated and a matrix was constructed. The program POPGENE (Yeh et al., 1997) was then used to calculate the divergence values (H), genetic divergences (G_{st}) and genetic distances among different clusters (Nei 1973, 1978). The G_{st} values were then used to estimate gene flow (N_m). The cluster genetic distance matrix obtained from an AMOVA (Excoffier et al., 1992) using the Popgene software was input into the SAHN and UPGMA modules of NTSYS to conduct a cluster analysis and generate a cladogram.

RESULTS AND DISCUSSION

Leaf morphology

The results of an analysis of variance of the leaf morphology data for all sampled trees are shown in Table 3. There were significant differences in leaf morphology among the *Cinnamomum* species ($p < 0.0001$). NTSYS was used to generate a cladogram from these data (Figure 2). Comparison of the cophenetic correlation coefficients of the distance matrix and the cladistic matrix yielded an *r*-value of 0.9461, indicating that the clusters derived from the leaf morphology data did not differ significantly from those derived from the genetic data. The cladogram derived from the leaf morphology data (distance coefficient = 40) clearly placed *Cinnamomum philippinense, Cinnamomum micranthum, Cinnamomum subavenium, Cinnamomum reticulatum* and *Cinnamomum kotoense* as independent species. *Cinnamomum japonicum* and *Cinnamomum pseudomelastoma* formed a cluster and *C. osmophloeum, Cinnamomum insularimontanum, Cinnamomum camphora, Cinnamomum brevipedunculatum* and *Cinnamomum macrostemon* formed a large and diverse cluster. We postulate that the species vary widely in leaf morphological characteristics in their natural communities, with many characters overlapping between species. Many *Cinnamomum* species have very similar leaf morphology. *C. micranthum*, *C. camphora* and *C. philippinese* have distinctive leaf textures or venation, but all other species exhibit similar ranges of variation. In particular, *C. insularimontanum, C. macrostemon, C. subavenium* and *C. osmophloeum* have highly variable leaf morphology, making their identification quite difficult in the field. Other plant parts, such as bud scales, petals or fruits are often required for positive identification.

Molecular relationships

ITS sequence analysis

The ITS sequences of the 12 endemic *Cinnamomum* species encompassed the 18S (3'), 5.8S and 26S (5') regions. *C. macrostemon* had the longest sequence (869 bp), while *C. camphora* had the shortest (813 bp). A total

Table 3. Quantitative characteristics of *Cinnamomum* leaves.

Taxon	N*	(1) Leaf length (cm)	(2) Leaf width (cm)	(3) Length /width ratio	(4) Angle of apex (°)	(5) Angle of base (°)	(6) Apex/base angle ratio
C. brevipedunculatum	23	7.22±1.39^{cd}	2.50±0.42^{cd}	2.89±0.48^{ab}	60.78±19.72^{bcd}	81.00±12.93^{a}	0.75±0.22^{cd}
C. camphora	24	8.69±1.75^{bc}	3.27±0.51^{bc}	2.69±0.47^{bc}	72.38±11.95^{ab}	77.42±13.14^{ab}	0.95±0.12^{abc}
C. insularimontanum	26	8.99±1.04^{bc}	2.69±0.44^{cd}	3.30±0.30^{ab}	56.59±10.94^{bcd}	71.19±16.34^{abc}	0.83±0.16^{bcd}
C. japonicum	17	8.08±1.06^{bcd}	3.04±0.70^{bcd}	2.77±0.64^{abc}	53.29±9.59^{cd}	61.71±10.65^{bc}	0.88±0.10^{abcd}
C. kotoense	20	13.99±3.16^{a}	4.88±1.01^{a}	3.17±0.72^{ab}	64.80±11.76^{bc}	71.25±13.08^{abc}	0.92±0.10^{abc}
C. macrostemon	27	8.43±1.10^{bcd}	2.50±0.64^{cd}	3.50±0.60^{a}	47.63±11.24^{d}	71.63±18.27^{abc}	0.69±0.09^{d}
C. micranthum	19	9.89±1.43^{b}	3.69±0.85^{b}	2.77±0.49^{abc}	84.37±15.00^{a}	81.21±8.80^{a}	1.02±0.14^{ab}
C. osmophloeum	24	8.11±1.28^{bcd}	3.23±0.89^{bc}	2.66±0.70^{bc}	47.67±9.33^{d}	59.04±12.63^{c}	0.85±0.11^{bcd}
C. philippinense	18	7.99±1.55^{bcd}	2.63±0.46^{cd}	2.88±0.45^{abc}	56.53±10.47^{bcd}	62.24±9.69^{bc}	0.94±0.15^{abc}
C. austro-sinense	18	13.36±2.75^{a}	4.72±1.00^{a}	2.90±0.41^{ab}	61.07±7.92^{bcd}	66.11±6.26^{abc}	0.94±0.08^{abc}
C. subavenium	19	7.47±0.74^{cd}	2.15±0.23^{d}	3.47±0.32^{a}	60.89±6.28^{bcd}	63.89±6.13^{abc}	0.95±0.08^{ab}
C. reticulatum	34	6.44±1.00^{d}	2.86±0.55^{bcd}	2.15±0.39^{c}	83.26±10.77^{a}	74.82±17.66^{abc}	1.07±0.23^{a}

*N: Number of samples.

of 72 sequences from the 12 species were aligned using the ClustalV algorithm in the program MegAlign. The total aligned sequence length was 885 bp.There were 293 variable nucleotide sites throughout the sequence, or 33.12% of the total. Among these, there were 238 parsimony informative sites or 26.89% of the total. The ratio of transitions to transversions (R) was 2.943. This value exceeds the expected value of 0.5, indicating that the endemic species of the genus in Taiwan are continuing to evolve (Graur and Li, 2000).

Using the BLAST search and sequence comparison functions provided by NCBI, Tsai (1990) has compared the 5.8S, 18S 3' end and 26S 5' end sequences of *Rhododendron kanehiraei*. These authors have also generated the entire 18S, 5.8S and 26S sequences of members of *Cinnamomum*, including a 57 bp 18S 3' end sequence, an 87 bp 26S 5' end sequence and a 162 bp 5.8S sequence (Table 4; Figure 4). We conducted 1000 bootstrap iterations in both the NJ and MP modes in MEGA v. 4.0. We then generated NJ (Figure 5) and MP (Figure 6) consensus dendrograms. Although, certain species exhibited closer interspecific kinships, we observed distinctive interspecific boundaries, indicating that the ITS sequences of the genus *Cinnamomum* contain significant interspecific differentiation. Thus, ITS sequences can successfully differentiate the species. The NJ method does not assume that all sequences have identical rates of evolution; therefore, the tips of the cladogram are uneven. Nevertheless, the consensus tree established by the NJ method largely agrees with that established by the MP method.

Summarizing the clustering results from the two methods, we found support for close relationships among certain species of the genus. These relationships are described as:

C. camphora and *C. micranthum cluster* (bootstrap support values of 99% for both the *NJ* and *MP* methods)

All data sources analyzed, including leaf morphology, ITS sequences and ISSR markers, support the position of these 2 species as a distinctive cluster apart from all other species. Due to the non-conservative evolutionary nature of the ITS region, sequencing analysis can often effectively differentiate species. Based on the ITS sequences, this cluster had the highest bootstrap support (99%) of all of the species clusters. An 11 bp deletion (between sites 496 and 506) was found only in these 2 species (Table 5).

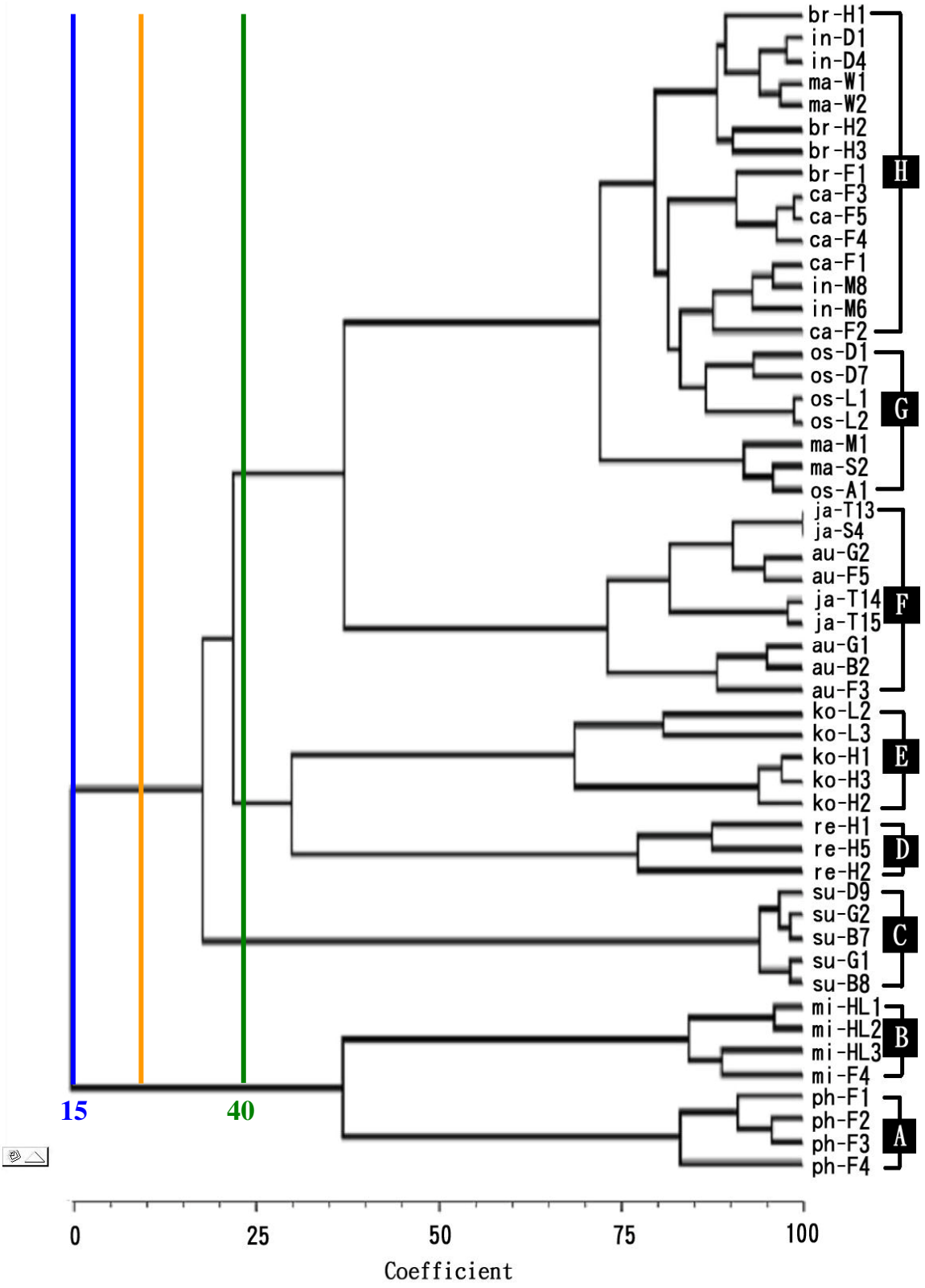

Figure 2. UPGMA dendrogram based on a phenetic analysis of leaf morphology (Cluster A: *C. philippinense*; B: *C. micranthum*; C: *C. subavenium*; D: *C. reticulatum*; E: *C. kotoense*; F: *C. C. pseudomelastoma* and *C. japonicum*; G: *C. osmophloeum*; H: *C. insularimontanum*, *C. macrostemon*, *C. camphora* and *C. brevipedunculatum*; refer to Table 1 for OTU codes).

Table 4. Comparison of *Cinnamomum* and *Oryza sativa* 5.8S sequences.

Parameter	Identities: 160/163 (98%) Gaps: 0/163 (0%)
Cinna.	ACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAA
Oryza	ACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAA
Cinna.	ATGCGATACCTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGC
Oryza	ATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGC
Cinna.	AAGTTGCGCCCGAGGCCATCCGGCCGAGGGCACGCCTGCCTGGGCGTCACGCCA
Oryza	AAGTTGCGCCCGAGGCCACTCGGCCGAGGGCACGCCTGCCTGGGCGTCACGCCA

Cinna: *Cinnamomum; Oryza*: *Oryza sativa.*

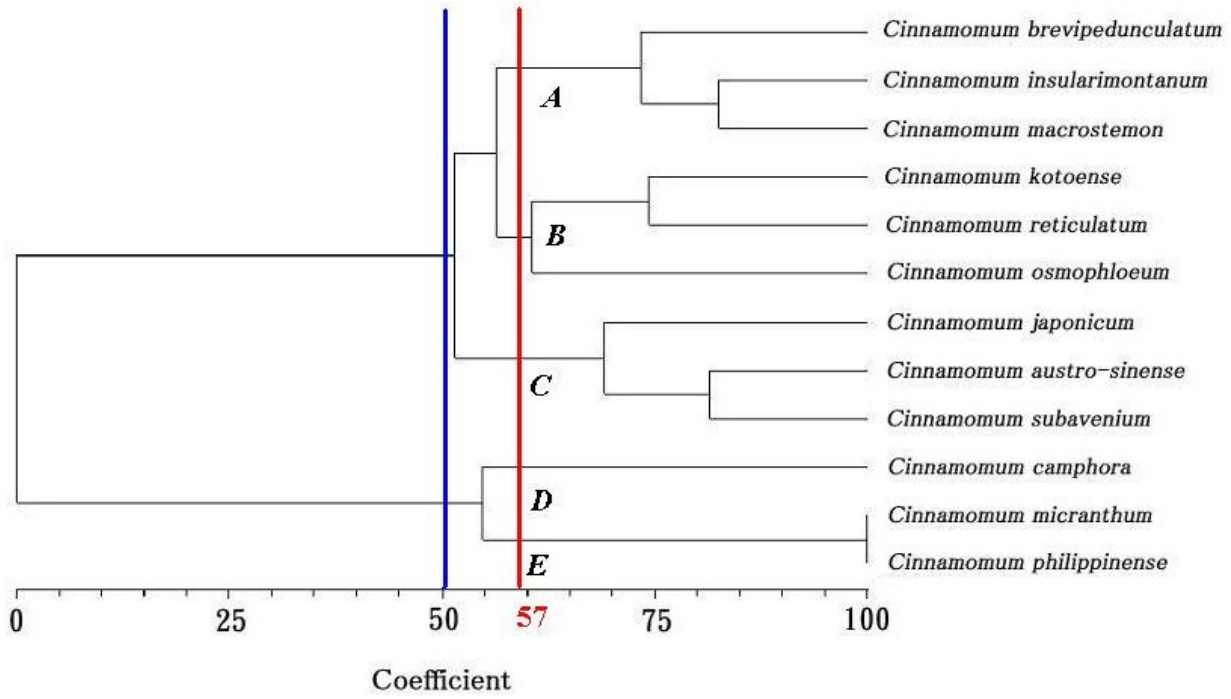

Figure 3. UPGMA dendrogram based on ISSR data. Two clusters are grouped at a distance coefficient of 50 (corresponding to sections *Camphora* and *Cinnamomum*). Five clusters are grouped at a distance coefficient of 57.

C. insularimontanum and C. macrostemon* cluster *and C. subavenium cluster* (bootstrap support values of 83% for *NJ* and *72%* for *MP)

C. insularimontanum and *C. macrostemon* formed a cluster, but this grouping received only 40% boot- strap support. When this cluster was expanded to include *C. subavenium,* however, the NJ bootstrap support reached 83%, indicating that *C. subavenium* is closely related to the former 2 species. The kinship between *C. macrostemon* and *C. insularimontanum* was also supported by the ISSR clustering results. Despite their being part of the same cluster, these 2 species exhibited notable differentiation, as indicated by their bootstrap support of only 40%. Thus, we infer that either *C. subavenium* is the common ancestral species of both *C. insularimontanum* or *C. macrostemon* or these 3 species arose from a common extinct ancestor. Subsequently, the species differentiated over the eons through geographic and reproductive isolation. *C. macrostemon* has a very narrow distribution in Taiwan, with only scattered natural occurrences. As a result, it may gradually undergo further speciation due to limited gene flow imposed by geo- graphic isolation. The other 2 species have wider and often overlapping distributions and are less affected by geographic isolation. However, *C. insularimontanum* flowers in April and May, while *C. subavenium* flowers in August and September of each year. Thus, these 2 species may be reproductively isolated, leading to speciation. *C. macrostemon* flowers in February to May of each year, overlapping with the flowering period of *C. insularimontanum*. Therefore, these 2 species might be able to exchange genes.

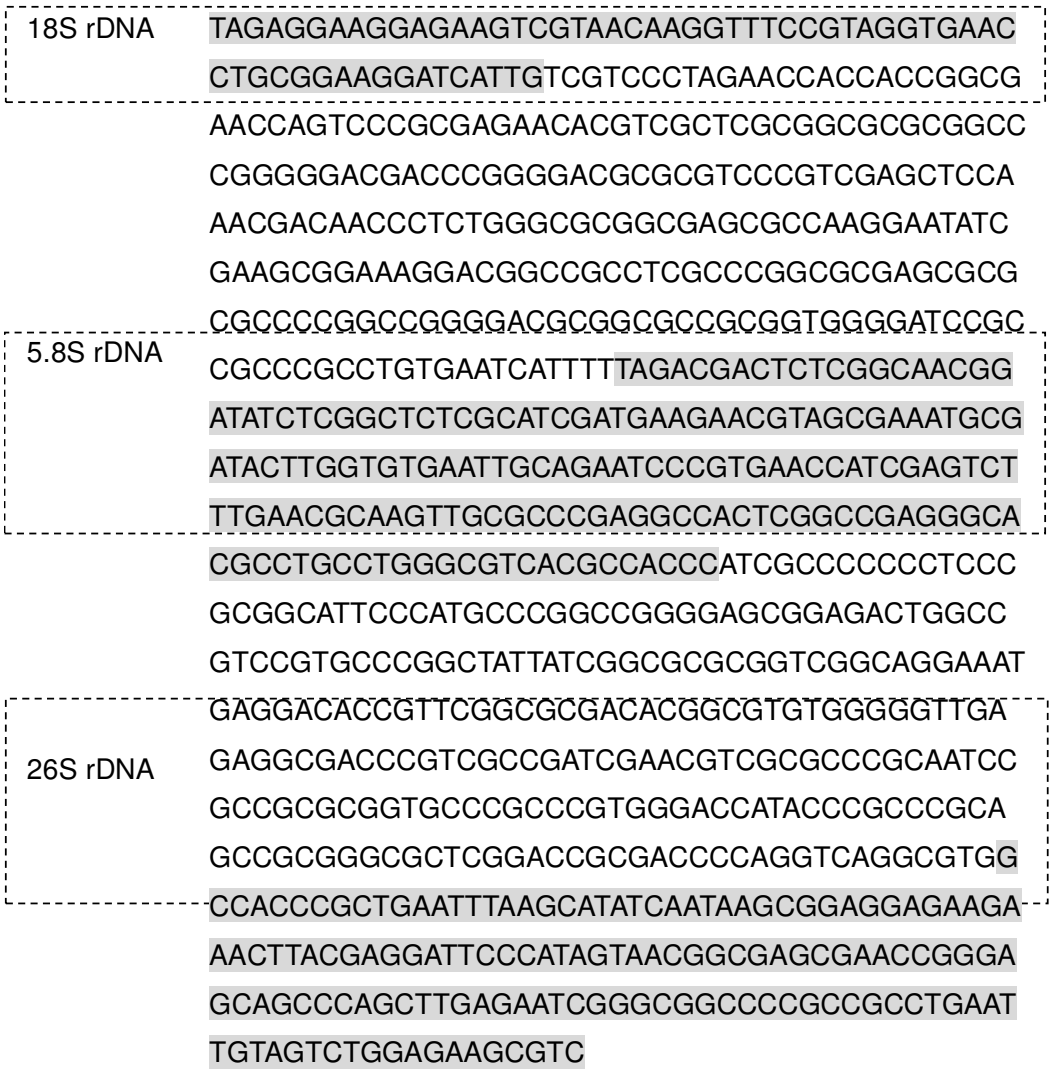

Figure 4. 18S, 5.8S and 26S rDNA sequence of *Cinnamomum*. Sequence C.br_H1_a is used here as an example. The shaded portions indicate the 18S, 5.8S and 26S rDNA regions, respectively.

***C. reticulatum, C. japonicum and C. pseudomelastoma cluster* (bootstrap support value of 78% with the *NJ* method)**

Within this cluster, the grouping of *C. reticulatum* and *C. pseudomelastoma* had a low NJ bootstrap support value of 52%, while the three-species cluster (including *C. japonicum*) received an NJ bootstrap value of 78%, suggesting that *C. japonicum* might be a common ancestor of the other 2 species. Notably, all 3 species in this cluster have been designated by the IUCN as critically endangered, endangered or threatened. *C. japonicum* is distributed only on Lanyu Island; *C. reticulatum* is found only in the lowland and costal forests of the Hengchun Peninsula; and *C. pseudomelastoma* is scattered throughout the montane regions of central and northern Taiwan. The rather divergent habitats of the 3 species suggest that geographic isolation might play an important role in their differentiation. In addition, both the ISSR and leaf morphology data tended to support the kinship between *C. pseudomelastoma* and *C. japonicum*.

C. osmophloeum, C. camphora, and C. micranthum cluster (bootstrap support values of 91% for NJ and 89% for MP)

Morphologically, *C. osmophloeum* resembles *C. insularimontanum* and *C. macrostemon.* However, the NJ and MP clustering methods both indicated that these species are surprisingly close relatives of *C. camphora* and *C. micranthum*. According to the trend in leaf venation, trinervate venation was derived from pinnate venation. Hence, the pinnate veins of C. *micranthum* and the

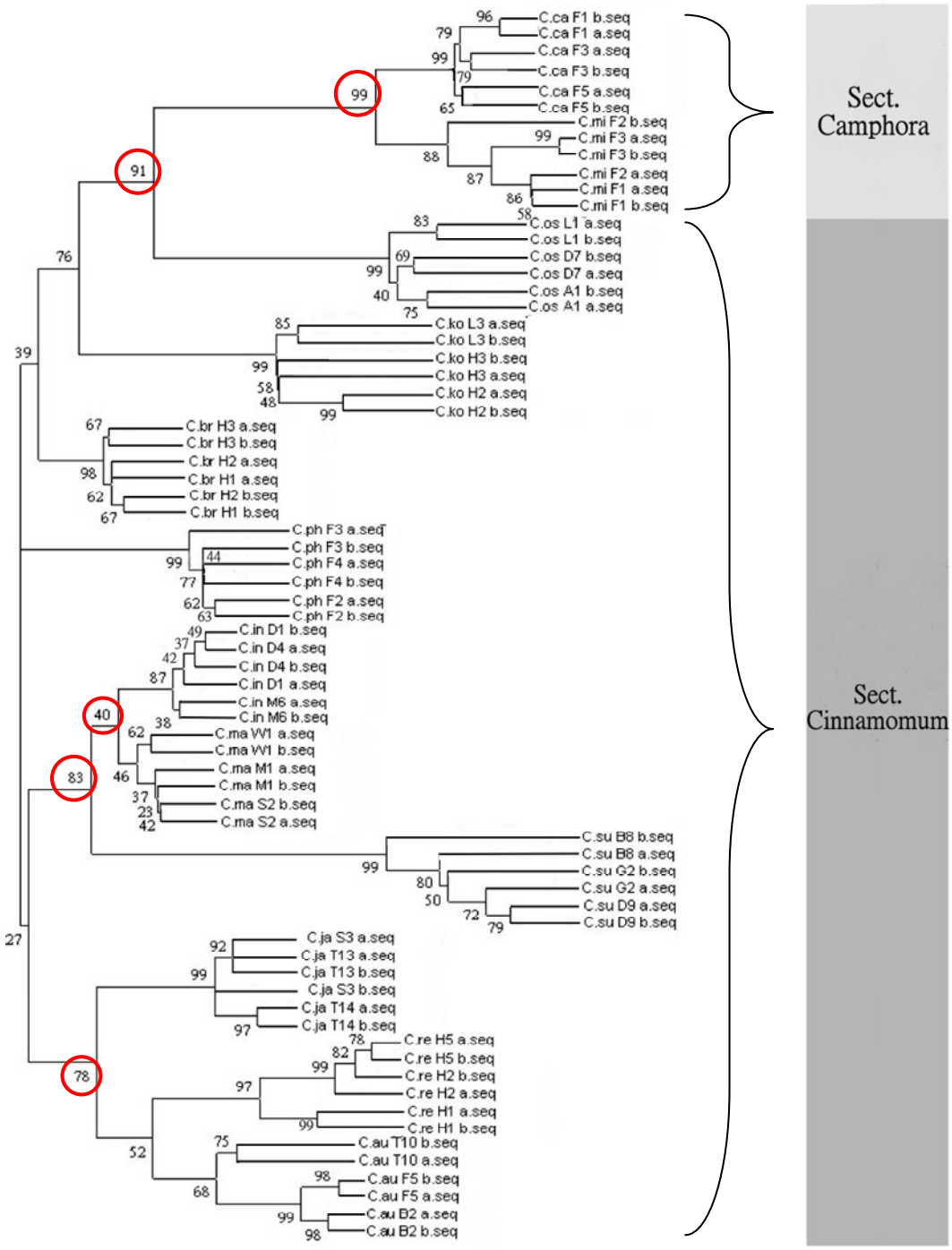

Figure 5. NJ consensus tree constructed based on ITS sequences.

presence of both pinnate and trinervate veins in *C. camphora* represent a more primitive cluster within the genus (Huang, 1984). We postulate that *C. osmophloeum* might be a transitional species between sections *Camphora* and *Cinnamomum*. *C. osmophloeum* is a common, widely distributed species that is often scattered in natural broadleaf forests at 400 to 1200 m in elevation (Hu, 1992). The species exhibits frequent genetic exchange with other species and thus, has a low degree of genetic differentiation. Ho (2006) has studied the genetic differentiation coefficients of *C. osmophloeum, C. insularimontanum* and *C. macrostemon*. The former species had a coefficient of

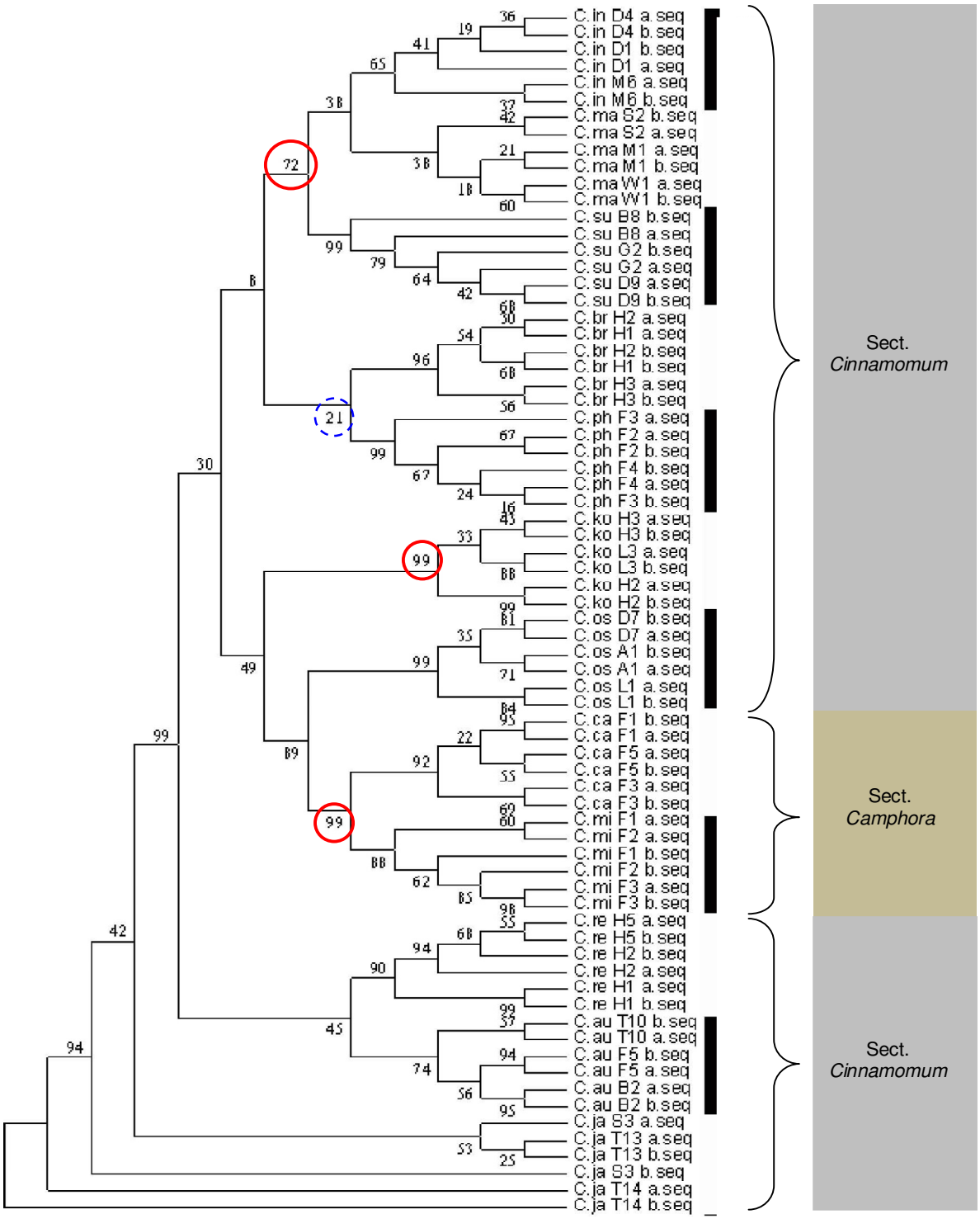

Figure 6. MP consensus tree constructed based on ITS sequences.

0.2837, while the latter 2 species had coefficients of 0.4988 and 0.4218, respectively, approaching those of selfing plants (0.51 on average). These observations reinforce the inferred speciation processes of *C. insularimontanum* and *C. macrostemon*. The high level of gene flow in *C. osmophloeum* might cause it to evolve more slowly than other members of section *Cinnamomum* and to retain more features of section *Camphora*.

Table 5. Specific deletions in *C. camphora* and *C. micranthum.*

C.br_H1_a	C**D**CGCGGCATTCC	C.mi_F1_a	C**DDDDDDDDDDD**C
C.br_H1_b	C**D**CGCGGCATTCC	C.mi_F1_b	C**DDDDDDDDDDD**C
C.br_H2_a	C**D**CGCGGCATTCC	C.mi_F2_a	C**DDDDDDDDDDD**C
C.br_H2_b	C**D**CGCGGCATTCC	C.mi_F2_b	C**DDDDDDDDDDD**C
C.br_H3_a	C**D**CGCGGCATCCC	C.mi_F3_a	C**DDDDDDDDDDD**C
C.br_H3_b	C**D**CGCGGCATTCC	C.mi_F3_b	C**DDDDDDDDDDD**C
C.ca_F1_a	C**DDDDDDDDDDD**C	C.os_A1_a	CGCGCGGCATTCC
C.ca_F1_b	C**DDDDDDDDDDD**C	C.os_A1_b	CGCGCGGCACTCC
C.ca_F3_a	C**DDDDDDDDDDD**C	C.os_L1_a	CGCGCGGCACTCC
C.ca_F3_b	C**DDDDDDDDDDD**C	C.os_L1_b	CGCGCGGCACTCC
C.ca_F5_a	C**DDDDDDDDDDD**C	C.os_D7_a	CGCGCGGCATTCC
C.ca_F5_b	C**DDDDDDDDDDD**C	C.os_D7_b	CGCGCGGCATTCC
C.in_D1_a	**DD**CGCGGCATTCC	C.ph_F2_a	CGCGGGGCATTCC
C.in_D1_b	CGCGCGGCATTCC	C.ph_F2_b	CCCGCGGCATTCC
C.in_D4_a	CGCGCGGCATTCC	C.ph_F3_a	CCCGCGGCATTCC
C.in_D4_b	CGCGCGGCATTCC	C.ph_F3_b	CCCGCGGCATTCC
C.in_M6_a	CGCGCGGCATTCC	C.ph_F4_a	CCCGCGGCATTCC
C.in_M6_b	CGCGCGGCATTCC	C.ph_F4_b	CCCGCGGCATTCC
C.ja_T13_a	CCCGCGGCATTCC	C.au_B2_a	CCCGCGGCATTCC
C.ja_T13_b	CCCGCGGCATTCC	C.au_B2_b	CCCGCGGCATTCC
C.ja_T14_a	CCCGCGGCATTCC	C.au_F3_a	CCCGCGGCATTCC
C.ja_T14_b	CCCGCGGCATTCC	C.au_F3_b	CCCGCGGCATTCC
C.ja_S4_a	CCCGCGGCATTCC	C.au_T10_a	CCCGCGGCATTCC
C.ja_S4_b	C**D**CGCGGCATTCA	C.au_T10_b	CCCGCGGCATTCC
C.ko_H2_a	C**D**CGCGGCATTCA	C.su_B8_a	CCCGCGGCATTCC
C.ko_H2_b	C**D**CGCGGCATTCA	C.su_B8_b	CGCGCGGCATTCC
C.ko_H3_a	C**D**CGCGGCATTCA	C.su_G2_a	CGCGGGGCATTCC
C.ko_H3_b	C**D**CGCGGCATTCA	C.su_G2_b	CGCGGGGCATTCC
C.ko_L3_a	C**D**CGCGGCATTCA	C.su_D9_a	CGTGCGGCATTCC
C.ko_L3_b	CGCGCGGCATTCC	C.su_D9_b	CGCGCGGCATTCC
C.ma_W1_a	CGCGCGGCATTCC	C.re_H1_a	**DDD**GCGGCATTCC
C.ma_W1_b	CGCGCGGCATTCC	C.re_H1_b	**DDD**GCGGCATTCC
C.ma_S2_a	CGCGCGGCATTCC	C.re_H2_a	**DDD**GCGGCATTCC
C.ma_S2_b	CGCGCGGCATTCC	C.re_H2_b	**DDD**GCGGCATTCC
C.ma_M6_a	CGCGCGGCATTCC	C.re_H3_a	**DDD**GCGGCATTCC
C.ma_M6_b	CGCGCGGCATTCC	C.re_H3_b	**DDD**GCGGCATTCC

D: Denotes base pair deletion.

Position of *C. philippinense*

C. philippinense stood apart from other species of the genus at the base of the tree according to the NJ analysis. In the MP analysis, this species grouped with *C. brevipedunculatum*; however, the bootstrap support value for this grouping was only 21%, suggesting that *C. philippinense* is more distantly relatedto other Taiwanese species of the genus. Historically, taxonomists have debated whether this species should belong to *Cinnamomum, Machilus* or *Persea*. In a study of leaf morphology, Huang (1984) has contended that members of the genus *Cinnamomum* have raised surface veins, whereas members of the other 2 genera have concave surface veins. Furthermore, chloroplast DNA sequences indicate that *C. philippinense* is more closely related to *Machilus* than to *Cinnamomum* and *Persea* and the swollen, obovate fruit of this species differs from those of other *Cinnamomum* species. The morphological similarities between *C. philippinense* and typical *Machilus* have led these authors to assert that the species should belong to the genus *Machilus* (Chang, 2005). Our leaf morphology, ISSR and ITS data indicate that *C. philippinense* is more closely related to the *C. camphora*

and *C. micranthum* cluster but is distinct from other species of the genus *Cinnamomum*.

ISSR clustering analysis

The Nei's gene diversity (H) of Taiwanese *Cinnamomum* was found to be 0.3535. The genetic divergence value (G_{st}) was as high as 0.9616 and the gene flow (N_m) parameter was only 0.02, suggesting strong interspecific differentiation. The ISSR cladogram produced from the POPGENE genetic matrix (Figure 3) indicates that at a distance coefficient of 0.50, the genus can be separated into 2 clusters. One cluster consists of *C. camphora, C. micranthum* and *C. philippinense*, while the other cluster encompasses the remaining species. These results are consistent with the division of Chinese *Cinnamomum* species into sections *Camphora* and *Cinnamomum* based on the morphological characteristics of flower petals, bud scale arrangements and glands (Li et al., 1983). According to these authors, both *C. camphora* and *C. micranthum* belong to section *Camphora*, while *C. philippinense* is not included in their scheme. Huang (1984) has also divided the species into 2 groups based on the presence or absence of distinctive sinuous leaf epidermal cells with uneven thickening of the cell walls. This group includes *C. brevipedunculatum, C. insularimontanum, C. kotoense, C. subavenium, C. japonicum, C. reticulatum* and *C. osmophloeum.* The second group, with smooth epidermal cells and without uneven cell wall thickenings, encompasses *C. camphora* and *C. micranthum*. Ou (1989) has studied the 12 Taiwanese *Cinnamomum* species through microscopic examination of their epidermal characteristics. Based on the unspecialized cells of the upper and lower epidermis and the bending of their hanging walls, the species can be grouped as having straight or slightly curved flanges versus varying degrees of undulating, U-shaped flanges. *C. camphora*, *C. micranthum* and *C. philippinense* all belong to the former group, in agreement with Huang's classification. Our leaf morphological observations also support the kinship of these 3 species. Five clusters split from the locus near distance coefficient 57 include:

C. micranthum and C. philippinense cluster:

This cluster is supported by the numerical analysis of the leaf morphology data. Liu et al. (1994) has separated these 2 species from the rest of the genus based on their pinnate leaf venation. The *C. philippinense* had been treated as the member of the genus *Machilus* or *Persea*. Ju et al. (2006) study by segments chloroplast DNA was used to analyze the relationship of *C. philippinense* with the species of the genera *C. innamomum*. *C. philippinense* had been treated as the member of the genus *Machilus* or *Persea*. Ju et al. (2006) study by segments chloroplast DNA were used to analyze the relationship of *C. philippinense* with the species of the genera *Cinnamomum*, *Machilus* and *Persea* in an attempt to resolve its taxonomic status, the result of chloroplast DNA analysis indicated that *C. philippinense* was closer to *Machilus* than to *Cinnamomum*.

C. camphora cluster

C. camphora forms a cluster of its own, indicating a more distant relationship with the other species, in support of genetic variation of *C. camphora* populations of Taiwan and surrounding areas (Ju and Wang, 2008).

C. subavenium, C. pseudomelastoma and C. japonicum cluster:

The numerical analysis of the leaf morphology data suggests that *C. pseudomelastoma* and *C. japonicum* are closely related. Liu et al. (1994) has noted that the bud scales of *C. japonicum* have a distinctive overlapping arrangement, thus, separating this species from the other 2, which lack bud scales. Our observations of their leaf morphology, however, indicate that all 3 species have trinervate veins that approach the leaf apex and that are 4/5 as long as the leaf. These trinervate veins are clearly raised on the back side of the leaf and are covered with light brown fuzz. Despite their distinctive external traits, the common leaf morphological characteristics and the ISSR results suggest that these 3 species are closely related.

C. osmophloeum, C. reticulatum and C. kotoense cluster

The numerical analysis of the leaf morphology data also indicates that *C. reticulatum* and *C. kotoense* form a cluster, consistent with the ISSR clustering. In a wood anatomical study of *Cinnamomum*, Chang (1995) has reported that *C. kotoense*, *C. osmophloeum* and *C. reticulatum* all lack terminal parenchyma bands but possess abundant longitudinal parenchyma that surrounds the vessels. In addition, traditional taxonomic keys often group *C. osmophloeum* and *C. kotoense* together because both lack bud scales and have smooth twigs and petioles (Liu et al., 1994).

C. insularimontanum, C. macrostemon and C. *brevipedunculatum cluster*

The numerical analysis of the leaf morphology data indicates that these 3 species form a cluster, but this

result is ambiguous. Based on their overlapping bud scales and caudate leaf apices, Liu et al. (1994) has suggested separating *C. macrostemon* and *C. insularimontanum* from the other species. However, Chang (1995) has grouped *C. brevipedunculatum* and *C. insularimontanum* together based on their wood anatomical feature of having longitudinal parenchyma tissue surrounding the vessels, while showing no aggregation.

Conclusions

Summarizing our leaf morphology, ISSR and ITS sequence analyses, we conclude that *C. micranthum* and *C. camphora* are closely related and that both have distinctive differences from the other Taiwanese species of the genus. These two species share an 11 bp deletion in their ITS sequences. The ITS analysis suggests that *C. insularimontanum, C. macrostemon* and *C. subavenium* are closely related and that the relationship between the first 2 species is supported by the leaf morphology, ISSR and ITS results. We postulate that *C. subavenium* might be a common ancestor of these 2 species or that all 3 species are descendents of a common extinct ancestor that gradually speciated over the eons through geographic and reproductive isolation. Additionally, the ITS results indicate that *C. japonicum, C. pseudomelastoma* and *C. reticulatum* are closely related. The relationship of the first 2 species is supported by the leaf morphology, ISSR and ITS results. According to the ITS results, *C. japonicum* might be the common ancestor of the other 2 species, which speciated as a result of their broad-scale geographic partitioning. Furthermore, the ITS results indicate that *C. osmophloeum* shares the more primitive pinnate venation of *C. camphora* and *C. micranthum*. The higher level of gene flow in the former species may have caused it to retain many genetic features of section *Camphora*. Our results further support the hypothesis that *C. osmophloeum* is a transitional species between sections *Camphora* and *Cinnamomum*.

REFERENCES

Chang CE (1976). Lauraceae. In: Li HL et al. (eds) Flora of Taiwan. Committee of the flora of Taiwan, Taipei. 2:406-420, p. 722.

Chang CE (2005). A review on the study of Lauraceae of Taiwan. Q. J. Chin. For. 38(4): 133-145.

Chang YS (1995). Systematic Taxonomy of Lauraceae plants in Taiwan using ylotomy. Master thesis of Institute of Forestry, National Chung Hsing University, p. 129.

Chen JQ, Li L, Li J, Li HW (2009). Bayesian Inference of nrDNA ITS Sequences from Machilus (Lauraceous) and Its Systematic Significance. Acta. Bot. Yunnanica. 31(2): 117-126.

Chiou ST, Yen JH, Fang CL, Chen HL, TY Lin (2007). Authentication of medicinal herbs using PCR-amplified ITS2 with specific primers. Planta Med. 73: 1421-1426.

Excoffier L, Smouse PE, Quattro JM (1992). Analysis of molecular variance inferred from metric distance among DNA haplotypes: Application to human mitochondrial DNA restriction data. Genetics. 131: 479-491.

Felsenstein J (1985). Confidence limits on phylogenies:An approach using the bootstrap. Evolution. 39: 783-791.

Fitch WM (1971). Toward defining the course of evolution: Minimum change for a specific tree topology. Syst. Zool. 20: 406-416.

Graur D, Li WH (2000). Fundamentals of Molecular Evolution. Sinauer Association, Inc., Sunderland, Massachusetts, USA. p. 481.

Higgins DG, Sharp PM (1989). Fast and sensitive multiple sequence alignment on a microcomputer. Comput. Appl. Biosci. 5: 151-153.

Ho KY (2006). Genetic Variation and Taxonomic Relationship of *Cinnamomum osmophloeum*, *C.macrostemon* and *C. insulari-montanum*(Lauraceae)in Taiwan. Bio. Formosa. 41(2): 93-102.

Hu TW (1992). The distribution, nutrition collection and database construction of *C. osmophloeum*. Taiwan For. Res. Inst. Book Series. 38: 2-6.

Huang HZ (1984). Leaf morphology of Lauraceae plants in Taiwan. Master thesis of Institute of Forestry, National Taiwan University, p. 149 .

Ju LP, Wang YN (2008). Genetic variation of *Cinnamomum camphora* populations of Taiwan and surrounding areas. Q. J. Chin. For. 41(4): 437-447

Ju LP, Wang YN, Pan FJ, JC, Yang JC, Lu SY (2006). Molecular phylogeny of *Cinnamomum philippinense* based on chloroplast dna sequences. Q. J. Chin. For. 39(2): 163-172.

Kobayashi N, Horikoshi T, Katsuyama H, Handa T, Takayanagi K (1998). A simple and efficient DNA extraction method for plants, especially woody plants. Plant Tis. Cul. Bio. 4: 72-80.

Kunjupillai V, Tsou CH (2008). Technical Report on the Molecular Phylogeny of Camellia with nrITS: The Need for High Quality DNA and PCR Amplification with Pfu-DNA Polymerase. Bot. Stud. 49(2): 177-188.

Lei YD, Tang XH, Tsering TS, Shi SH, Zhong Y (2004). A preliminary phylogenetic analysis of some Rhodiola species based on nrDNA ITS sequences. J. Gen. Mol. Bio. 15(3): 190-197.

Li J, Li XW (2004). Advances in Lauraceae systematic research on the world scale. Acta. Bot. Yunnanica. 26(1): 1-11.

Li L, Li J, Conran JG, Li XW (2007). Phylogeny of *Neolitsea* (Lauraceae) inferred from Bayesian analysis of nrDNA ITS and ETS sequences. Plant Syst. Evol. 269: 203-221.

Li SK, Yang HP, Li SW, Twei HB, Chao LL, Bai PY, Huang PH, Wei FN, Wei YT (1983). The Lauraceae family. In: Cheng WJ (eds) Tree Flora of China Chin. For. Publ. Beijing, 1: 735-770.

Li ZM, Li J, Li XW (2006). Polyphyly of the genus *Actinodaphne* (Lauraceae) inferred from the analysis of nrDNA ITS and ETS sequences. Acta Phytotaxon. Sin. 44(3): 272-285.

Liu YC, Lu FY, Ou CH (1994). Trees of Taiwan (Ⅱ). Nat. C. H. Univ. Monographic Publication. 7: 107-112.

Nei M (1973). Analysis of gene diversity in subdivided population. Proc. Natl. Acad. Sci. USA. 70: 3321-3323.

Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics. 89: 583-590.

Ou CH (1989). Microscopic morphology study of leaf dermis of Lauraceae plants in Taiwan. Ph.D. thesis of Institute of Botany, Nat. C. H. Univ. p. 186.

Rohlf FJ (1993). NTSYS-pc numerical taxonomy and multivariate analysis system. Applied Biostatistics, New York, p. 256.

Saitou N, Nei M (1987). The Neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596-1599.

Tsai CC (1990). Nucleotide Sequence of Internal Transcribed Spacer Region between 18S and 26S Ribosomal Genes from *Rhododendron kanehirai*. Taichung Dist. Agric. Improv. Res. Re. 63: 1-11.

Xiao WE, Cao HL, Jlang YS, Ye WH, Ge XJ, Li F (2008). Population genetic structure of *Camellia nitidissima* (Theaceae) and conservation implications. Bot. Stud. 49: 147-153.

Yeh FC, Yang RC, Boyle TBJ, Ye ZH, Mao JX (1997). Popgene, the user-friendly freeware for population genetic analysis. Molecular Biology and Biotechnology Center, University of Alberta, Canada.