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A comparison of nrDNA internal transcribed spacer (ITS) loci for phylogenetic inference and authentication among *Cinnamomum osmophloeum* and related species in Taiwan

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***Cinnamomum osmophloeum*, an indigenous species of Taiwan, can be utilized for valuable products such as a food, a spice and a traditional Chinese medicine. This study compares the ribosomal DNA (nr DNA) internal transcribed spacer (ITS) sequence of *C. osmophloeum* to that of several other species with similar external morphology, such as *Cinnamomum burmannii, Cinnamomum insularimontanum*, *Cinnamomum macrostemon* and *Cinnamomum subavenium*. Phylogeny of ITS sequences shows that *C. osmophloeum* is more closely related to *C. burmannii* than the other species, while *C. insularimontanum, C. macrostemon*, and *C. subavenium* are phylogenetically relevant to each other. By comparing ITS sequence between *C. osmophloeum* and *C. burmannii*, specific primers were designed for the multiplex-PCR to differentiate them. Based on ITS sequence differences, all tested *Cinnamomum* spp. can be properly authenticated. A 125 bp band specific for *C. osmophloeum* and a 204-bp *C. burmannii*-specific band were successfully amplified by polymerase chain reaction (PCR) using the respective primers described above. The two species then can be identified at the molecular level according to the sizes of their respective PCR products as determined by gel electrophoresis.**

Key words: *Cinnamomum osmophloeum*, internal transcribed spacer (ITS), phylogenetic, multiplex-polymerase chain reaction (PCR).

INTRODUCTION

There are approximately 250 species in the genus *Cinnamomum*. The plants are primarily found in tropical Asia, subtropical areas and Australia (Liu et al., 1994). However, only 12 *Cinnamomum* species are distributed

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Abbreviations: nr DNA, Ribosomal DNA; **ITS**, internal transcribed spacer; **CTAB**, cetyl trimethyl ammonium bromide; **PCR**, polymerase chain reaction; **NCBI**, National Center for Biotechnology Information; **NJ**, neighbor-joining method; **MP**, maximum parsimony; **BLAST**, basic alignment search tool.

Figure 1. The sample collection sites in this study.

in Taiwan, *Cinnamomum brevipedunculatum*, *Cinnamomum camphora*, *Cinnamomum insularimontanum*, *Cinnamomum japonicum*, *Cinnamomum kotoense*, *Cinnamomum macrostemon*, *Cinnamomum micranthum*, *Cinnamomum osmophloeum*, *Cinnamomum philippinense*, *Cinnamomum austrosinense*, *Cinnamomum subavenium* and *Cinnamomum reticulatum*. Typical diagnostic morphological characteristics of species in the *Cinnamomum* genus include three-branched leaf veins and plump cup-like hypocarps. Presence and arrangement of bud scales and the presence of trichomes on the bud scales, inflorescence and perianth, are also used as common morphological diagnostic characters for the species delimitation within the *Cinnamomum* genus (Liu et al. 1994; Liao, 1996). Studies on the taxonomy of Taiwanese endemic *Cinnamomum* plants have mainly focused on external morphology or the micro-anatomical phenotypes of lignum and leaves (Huang, 1984; Ou 1989; Chang, 1995).

C. osmophloeum has been widely adopted by forest farmers in Taiwan because it is rich in cinnamaldehyde, a major constituent of *Cinnamomum cassia,* the cinnamon of commerce, and has excellent natural anti-bacterial activity, anti-cancer and other pharmacological properties (Chang et al., 2001, Cheng et al., 2004, Cheng et al., 2008, Chen et al., 2010). *C. burmannii* originates in southern China and the East Indian archipelago and is a newly naturalized species in Taiwan (Tseng et al., 2008). As the two species are similar in appearance, they are not easily differentiated by their phenotypes, which often lead to economic losses because of planting errors by forest farmers.

Various types of DNA markers provided by modern molecular biology can be used for delimitation of species that are not easily distinguished based on morphology. Ribosomal DNA (nr DNA) internal transcribed spacer (ITS) sequences can be employed for a broad range of purposes including species identification, phylogenetic studies and the study of evolution (Lau et al., 2001; Alvarez and Wendel, 2003; Chen et al., 2009). ITS of nr DNA also has been used in molecular authentication technique by virtue of the presence of at least one single nucleotide difference in this region between similar species (Park et al., 2005; Chiou et al., 2007; Wang et al., 2010).

This study uses nuclear 5.8S rRNA and the ITS sequences as genetic markers to investigate the differences and phylogenetic relationships among *C. osmophloeum* and related similar species, including *C. osmophloeum*, *C. insularimontanum*, *C. macrostemon*, *C. subavenium* and *C. burmannii*. Specific primers were synthesized after comparing variations in the ITS2 sequences between the indigenous *C. osmophloeum* and exotic *C. burmannii*, and they were successfully used to differentiate the two species at the molecular level in a timely fashion.

MATERIALS AND METHODS

Source of samples and their treatment

C. osmophloeum, *C. insularimontanum*, *C. macrostemon*, *C.*

Table 1. Fifteen *Cinnamomum* spp. samples collected in this study and nine *Cinnamomum* spp. available from GenBank used for phylogenetic analysis.

Species	Collection site	Abbreviation (GenBank accession)
(A) *Cinnamomum* spp. samples collected in the present study		
C. osmophloeum	De-fu-lan Mountain (Taichung County)	os-d (GU598524)
	Lan-tan (Chiayi County)	os-g (GU598525)
	San-jiao South Mountain (Tainan County)	os-s (GU598526)
	Ping-lin (Taipei County)	os-t (GU598527)
C. burmannii	Lan-tan (Chiayi City)	bu-l (GU598530)
	She-kou (Chiayi County)	bu-s1 (GU598531)
	She-kou (Chiayi County)	bu-s2 (GU598532)
C. insularimontanum	Dong-pu (Nantou County)	in-d (GU598533)
	Mei-lan (Kaohsiung County)	in-m (GU598534)
	Tun-ye-sheng-tai Mountain (Hualian County)	in-t (GU598535)
C. macrostemon	Mo-tuo-wan Mountain (Hualien County)	ma-m (GU598521)
	Wu Peak (Hsinchu County)	ma-w (GU598522)
	Ba-dao-er Mountain (Taipei County)	ma-b (GU598523)
C. subavenium	Shan-ping (Kaohsiung County)	su-c (GU598528)
	Lu Mountain (Kaohsiung County)	su-l (GU598529)
(B) Sequence from GenBank		
C. amoenum	Parana, Curitiba, Brazil	(FM957801)
C. burmannii	Berlin, Germany	(FM957802)
C. daphnoides	Berlin, Germany	(FM957803)
C. duartianum	Brazil	(FM957804)
C. japonicum	N/A	(AF272263)
C. oleifolium	N/A	(AF272264)
C. pittosporoides	Yunnan, China	(DQ124269)
C. quadrangulum	N/A	(AF272265)
C. verum	N/A	(AF272267)
Neolitsea dealbata	Queensland, Australia	(DQ124277)

*Specimen were used in multiplex-PCR experiment in identification of *C. osmophloeum* and *C. burmannii.*

subavenium and *C. burmannii* were collected from a wide range of areas in Taiwan including the northern, central, southern, and eastern regions (Figure 1). The leaves were dehydrated, aliquotted into air-permeable bags, and stored dry in sealed containers. Leaf samples of the different species used in this study were collected from Taiwan. Species identification was performed by Dr. Fu-Yuan Lu from the Department of Forestry and Nature Resources at the National Chiayi University. The leaves were dehydrated, numbered and stored at the Department of Forestry and Nature Resources at the National Chiayi University. Table 1 shows the various species names, collection areas and GenBank accession numbers of the samples used in this study.

DNA extraction, polymerase chain reaction, and sequencing

Genomic DNA was extracted according to the modified cetyl trimethyl ammonium bromide (CTAB) method (Kobayashi et al., 1998) with minor modification. DNA extracted from tested materials was amplified using a pair of primers specific for nuclear nr DNA (partial 18S+ITS1+5.8S+ITS2+partial 26S) by polymerase chain reaction (PCR). The total volume of each PCR reaction was 25 µl, containing 1.5 µl of DNA, 2 µl of dNTPs (2.5 mM), 2.5 µl of 10X Taq buffer, 2 µl of DMSO, 2.1 µl of Mg^{++}, 0.2 µl of Taq, 1 µl of each primer, and 13.7 µl of ddH_2O. PCR reactions were carried out under the following conditions: denaturature at 94°C for 5 min, and then 94°C for 30 s, 58°C for 50 s (annealing), and 72°C for 1 min (extension), for 30 cycles, and finally 72°C for 10 min for the final extension. The complete ITS1-5.8S-ITS2 fragments of tested *Cinnamomum* spp. were amplified using the forward primer X-12: 5′-TAGAGGAAGGAGAAGTCGTAA-3′, located approximately 50 nucleotides to the 3′ terminus of the 18S rDNA sequence and the reversed primer A25-1: 5′-GACGCTTCTCCAGACTACAAT-3′ located approximately 160 nucleotides to the 5′ terminus of the 26S rDNA sequence. The set of primers were designed by Chiou et al. (data not published) based on the conservative sequences of medicinal plants in Chinese pharmacopoeia.

PCR products were separated by electrophoresis on a 1.2% agarose gel in 0.5 x TBE buffer. Following electrophoresis, the gel was stained, visualized and to verify the size of the amplified DNA fragment of interest was correct. Each of the PCR products was then direct sequenced at least three times to confirm the correctness at Genomics BioSci and Tech (Taipei, Taiwan).

Muplex-PCR in authenticating of *C. osmophloeum* and *C. burmannii*

Primer pairs set specific for *C. osmophloeum* and *C. burmannii* were designed based on a single nucleotide difference found after comparing their ITS2 sequence (Figure 2). The sequences of the

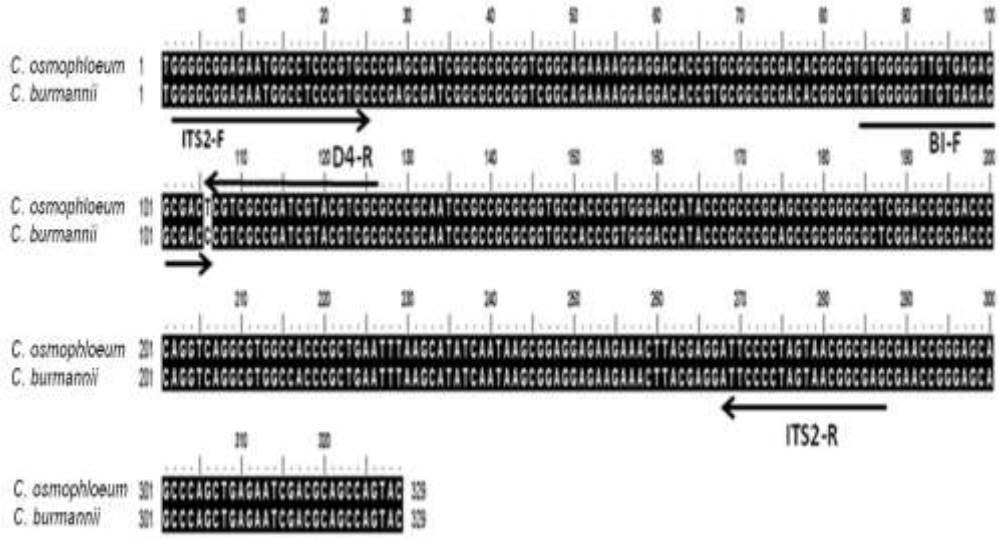

Figure 2. Design scheme for primers ITS2-F, D4-R, BI-F, and ITS2-R in the ITS2 region.

Table 2. Statistics of the positions of rDNA segregation and mergence.

Species	rDNA lengths (bp)				
	ITS1	5.8S	ITS2	ITS1+ITS2	Total length
C. osmophloeum	202	169	241	443	612
C. insularimontanum	199	169	245	444	613
C. macrostemon	202	169	245	447	616
C. subavenium	200	169	240	440	609
C. burmannii	202	169	240	442	611

primer pairs were as follows: ITS2-F: 5′GGG GCG GAG AAT GGC CTC CCG TGC-3′; D4-R: 5′-GCG ACG TAC GAT CGG CGA CTA-3′; BI-F: 5′-GTG GGG GTT GTG AGA GGC GAT C-3′; ITS2-R 5′-CTC GCC GTT ACT AGG GGA AT-3′. The total volume of each PCR reaction was 25 µl, containing 1.5 µl of DNA, 2.0 µl of dNTPs (2.5 mM), 2.5 µl of 10X Taq buffer, 2 µl of DMSO, 2.1 µl of Mg^{++}, 0.2 µl of Taq, 1 µl of primers (contains 0.25 µL of ITS2-F 'D4-R 'BI-F and ITS2-R), and 13.7 µl of ddH_2O. PCR reactions were carried out under the following conditions: denaturing at 96°C for 12 min, and then 95°C for 30 s, 58°C for 50 s (annealing), and 72°C for 1 min (extension), for 35 cycles, and finally 72°C for 10 min for the final extension.

The PCR products were subjected to an agarose gel electrophoresis and the gel was stained with ethidium bromide. After obtaining an image of the gel, the bioimaging analysis software ImageJ v1.41 (http://rsb.info.nih.gov/ij/index.html) was employed to translate band intensity to an intensity plot amenable to analysis. The DNA bands corresponding to *C. osmophloeum* and *C. burmannii* sources were cut from the agarose gel using sterile razor blades, and the extracted PCR products were submitted to the Genomics BioSci and Tech (Taipei, Taiwan) for DNA sequencing. Results obtained from the DNA sequencing were compared to sequences in the National Center for Biotechnology Information (NCBI) database using the BLAST software to determine whether they were the desired sequences.

Sequence analysis

The DNA sequence alignment was conducted using the Clustal V program provided in the MegAlign (Dnastar, Lasergene), then adjusted manually. Unique haplotypes were used for the following analyses. To compare the frequency and ratio of base transition and transversion, the nucleic acid base composition in the aligned sequences was analyzed using the MEGA version 4.0 (Kumar et al., 2008). The best-fitting model for the aligned sequences was determined using hierarchical likelihood ratio test performed by the program Modeltest 3.7 (Posada and Crandall, 1998). The selected substitution model was then adopted in the reconstruction of phylogeny by the neighbor-joining method (NJ) (Saitou and Nei, 1987), conducted by PAUP* 4.0b10 (Swofford, 2003). The parsimony analysis was conducted in PAUP by heuristic search under TBR branch swapping, random taxa addition of 500 replicates and MulTrees option in effect. Gaps within the alignment were treated as missing. The bootstrap analysis was performed 1000 times to test the consistency of the nodes of lineage branches in the tree. *Neolitsea dealbata* was selected as an outgroup because of its close relationship with *Cinnamomum* (Li et al., 2008).

RESULTS AND DISCUSSION

Comparison of the ITS sequence

Fifteen (15) *Cinnamomum* spp. samples of five species were collected and analyzed in this study (Table 1). Table 2 presents the comparison results of the ITS sequences among tested materials. The PCR amplified ITS

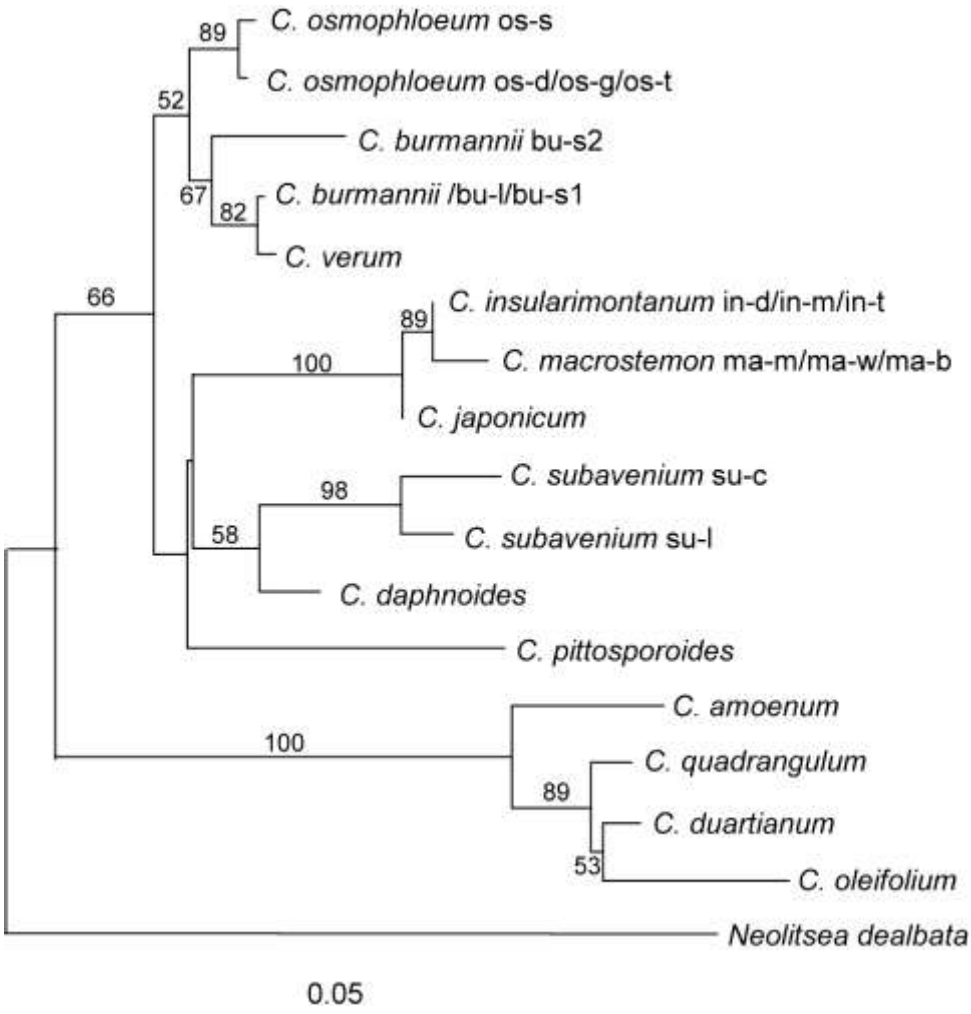

Figure 3. Neighbor-joining tree of 15 native Taiwan *Cinnamomum* species based on ITS fragment. Numbers beside the branches refer to the percentage of the bootstrap values of 1000 replicates. Bootstrap value less than 50% was not shown. See Table 1 for the abbreviations of samples.

sequences fragments were about 610 bps in length (ranged between 609 and 612 bp). Different species displayed variability in the length and composition of their ITS sequences. In this study, the length of ITS sequences (ITS1 + ITS2) ranged between 440 and 447 bp. The 5.8S rDNA gene sequences of the five species in this study are 169 bp in length, which is a relatively conserved region with 99.6% similarity (data not shown).

Phyletic analysis

The alignment provided 579 bp for phylogenetic analyses (data not shown). Eight haplotypes, each with unique sequence, were found from 15 samples. Individuals of the same haplotype belong to the same species regardless of their geographic origins. The best-fitted substitution model selected by Modeltest was HKY with gamma shape parameter alpha = 0.2853. This model was then used for NJ analysis. Neighbor-joining tree (Figure 3) showed high bootstrap supports for terminal nodes, but the supports for some deep nodes were low. Hence, the ITS sequence is suitable for the delimitations at species level. High intra-specific similarities were found, without splits within the same species. The constructed tree topology indicated that *C. osmophloeum* is relatively similar to the two foreign species, *C. burmannii* and *C. verum*. This agrees to morphological characters. The two endemic species, *C. insularimontanum* and *C.macrostemon*, were phylogenetically close to the Japanese species *C. japonicum*.

The heuristic searches resulted in 10 trees of equally maximum parsimony (MP) with a tree length of 122 (CI = 0.836, RI = 0.854). The 50% majority-rule consensus tree of the 10 MP trees was reconstructed (Figure 4). Terminal nodes were supported by high bootstrap values and showed the same topology as the NJ tree. According

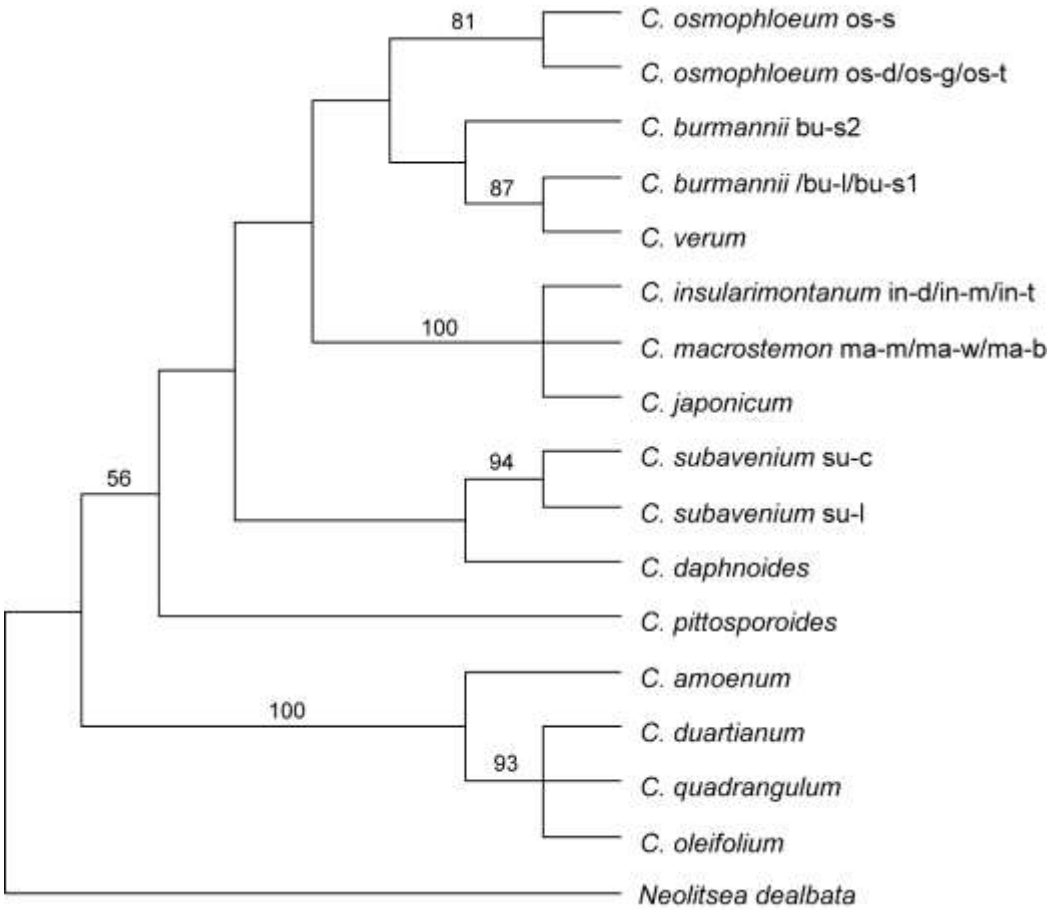

Figure 4. 50% majority-rule consensus maximum parsimonious tree of 15 native Taiwan *Cinnamomum* species based on ITS fragment. Numbers beside the branches refer to the percentage of the bootstrap values of 1000 replicates. Bootstrap value less than 50% was not shown. See Table 1 for the abbreviations of samples.

to the morphological classification of plants in the genus *Cinnamomum*, *C*. *insularimontanum* and *C*. *macrostemon* can be grouped together based on the external morphology of the bud. *C*. *insularimontanum* and *C*. *macrostemon* were grouped into the same clade in the phylogenetic tree constructed in this study.

Variability of ITS fragments between *C. osmophloeum* and its closely related species

The ITS fragments in *C. osmophloeum* samples collected from different geographic regions are highly similar to each other (Figures 3 and 4), indicating low genetic divergence of the ITS fragments within the species. Hence, the use of ITS sequence for molecular identification at species level is reliable for this species throughout Taiwan. The variability of ITS fragments between *C. osmophloeum* and *C*. *burmannii* was the lowest of all species compared, with proportional distances between 0.009 and 0.016 (Table 3).

Specificity of PCR amplification of the *C. osmophloeum* and *C. burmannii* DNAs using specific primer pairs

As shown in Figure 5, all 6 samples yielded a 286-bp band, which was amplified with the ITS2-F/ITS2-R primer pair. As this band was common for both species, it can be used to determine if an unknown sample is either *C. osmophloeum* or *C. burmannii* (Figure 5a). A subjective analysis of band intensity by the naked eye could easily lead to errors when reading obscured bands. ImageJ is a bioimaging analysis software that quantifies the bands. We employed it to convert individual bands on the gel image into a more objective intensity plot (Figure 5b). As shown in Figure 5a, the two *Cinnamomum* species shared a common peak at 286 bp. Furthermore, *C. osmophloeum* gave a specific peak at 125 bp while *C. burmannii* exhibited a specific peak at 204 bp. Note worthily, the specific peak from *C. osmophloeum* was much more intense than did that of the *C. burmannii*.

Three bands from Figure 5a were excised from the

Table 3. Proportional distance between haplotypes of *Cinnamomum* spp. and outgroup based on ITS sequences.

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
C. osmophloeum os-s																
C. osmophloeum os-d/os-g/os-t	0.002															
C. burmannii bu-l/bu-s1	0.011	0.009														
C. burmannii bu-s2	0.016	0.014	0.012													
C. insularimontanum in-d/in-m/in-t	0.025	0.025	0.022	0.027												
C. macrostemon ma-m/ma-w/ma-b	0.029	0.029	0.025	0.031	0.004											
C. subavenium su-c	0.027	0.027	0.030	0.036	0.036	0.040										
C. subavenium su-l	0.023	0.023	0.027	0.032	0.034	0.038	0.011									
C. amoenum	0.044	0.044	0.048	0.053	0.062	0.066	0.060	0.057								
C. daphnoides	0.017	0.017	0.019	0.022	0.022	0.026	0.022	0.015	0.054							
C. duartianum	0.045	0.045	0.047	0.053	0.063	0.066	0.060	0.057	0.019	0.058						
C. japonicum	0.023	0.023	0.020	0.025	0.002	0.005	0.034	0.032	0.061	0.020	0.061					
C. oleifolium	0.053	0.053	0.055	0.061	0.071	0.074	0.068	0.065	0.027	0.065	0.015	0.069				
C. pittosporoides	0.031	0.031	0.034	0.038	0.036	0.040	0.041	0.041	0.059	0.026	0.058	0.034	0.067			
C. quadrangulum	0.045	0.045	0.048	0.053	0.063	0.067	0.061	0.057	0.019	0.058	0.008	0.061	0.015	0.059		
C. verum	0.009	0.011	0.002	0.014	0.023	0.027	0.030	0.027	0.048	0.019	0.047	0.022	0.055	0.034	0.048	
Neolitsea dealbata	0.060	0.058	0.057	0.062	0.061	0.064	0.062	0.062	0.079	0.063	0.069	0.059	0.071	0.066	0.067	0.058

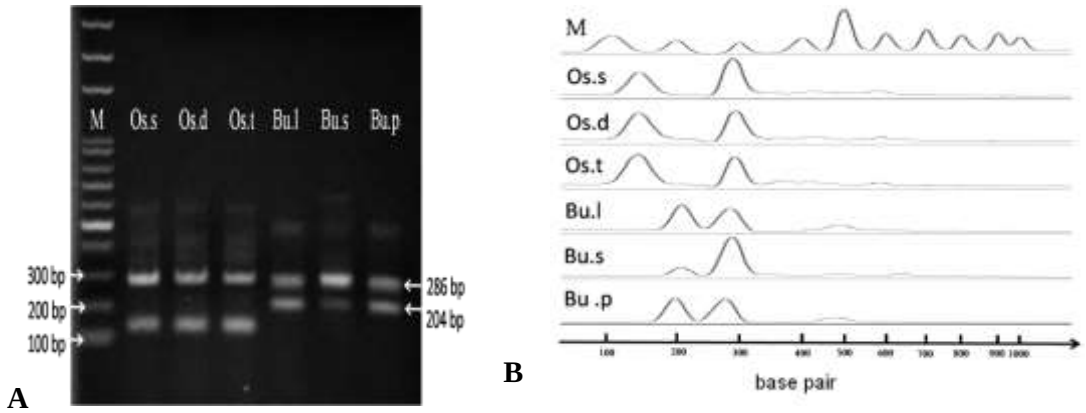

Figure 5. Electrophoretic analysis of PCR products using primers ITS2-F, D4-R, BI-F, and ITS2-R. **(a)** Agarose gel image. **(b)** BioImaging analysis of the electrophoresis gel image using ImageJ. M signifies 100-bp DNA markers; see also Table 1 for the abbreviations of samples.

agarose gel and subjected to sequencing to confirm the specificity of the bands. Sequencing results revealed that the 286, 204 and 125 bp bands were consistent with targeted sequences between primer pairs ITS2-F/ITS2-R, B1F/ITS2-R, and ITS2-F/D4-R, respectively. Moreover, the three obtained sequences were compared to entries in the NCBI database using the Basic Alignment Search Tool (BLAST) software to confirm that they were generated from the targeted sequences (data not shown).

This study describes a multiplex-PCR method to amplify PCR products of different lengths followed by agarose gel electrophoresis to resolve the bands. After staining and imaging the gel, molecular authentication could be performed based on specific bands from different species. Park et al. (2005) have successfully designed specific primer pairs based on a single nucleotide difference within the 5.8S region and effectively characterized Korean ginseng and other closely related species using multiplex-PCR.

Sequence of the ITS2 region in *C. osmophloeum* and *C. burmannii* is nearly identical, except for a single nucleotide difference at 106 bp where a T is presents in *C. osmophloeum* and a C in *C. burmannii* (Figure 2). The characteristic 125-bp band of *C. osmophloeum* could be specifically amplified with the ITS2-F/D4-R primer pair based on the single nucleotide difference. Similarly, the BI-F/ITS2-R primer pair can amplify the 204-bp band specific to *C. burmannii*. The ITS2-F/ITS2-R primer pair was designed based on the common DNA sequence in the ITS2 region of the two species; therefore, the pair could amplify the 286-bp band specific to both. The intensity of the DNA bands amplified by the ITS2-F/D4-R primer pair was higher than that of the bands amplified with the BI-F/ITS2-R primer pair, indicating that the current PCR condition was optimized for the *C. osmophloeum* DNA amplification. This high level of detection met the quarantine requirement and aid in species identification.

In order to examine whether accurate analytical results could be obtained when the primer pairs were used on the indigenous *C. osmophloeum* and exotic *C. burmannii* samples collected from different regions, we performed reproducibility experiments as well. The results show that the primer pairs could accurately amplify the characteristic bands of both species (data not shown). *C. osmophloeum*, which belongs to the same genus as *C. burmannii*, is a precious native forest resource. In recent years, *C. burmannii* has been imported and extensively used for environmental greening disguised as *C. osmophloeum* by some dishonest seed industry perpetrators.

As the two species are difficult to differentiate, and often cause confusion in their identification. A further complication was that if the bogus exotic species was used to extract its leaf essential oil besides serving the greening purpose; then the difference in its chemical compositions could also cause havocs in marketplace. There is a need for the study of its chemical races and compositions, and above all, to seriously assess the impacts of this exotic species on the local forest ecosystems.

Conclusions

The indigenous *C. osmophloeum* was efficiently and correctly distinguished from other related similar species in this study. The ITS fragments from five species revealed considerable stability demonstrating no significant variability linked to regional location. A fast molecular authentication technique was developed for distinguishing the indigenous *C. osmophloeum* from exotic *C. burmannii* by using a designed specific primer pairs to generate distinguishing bands via electrophoresis without resorting to sequencing, thereby saving time and money. The results from our study indicate that ITS sequences not only can be successfully used for the evolution studies but also for the delimitation of closely related similar *Cinnamomum* spcies which occurred in Taiwan.

Conflict of interests

The authors did not declare any conflict of interest.

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