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Genetic Identification of *Cinnamomum* Species Based on Partial Internal Transcribed Spacer 2 of Ribosomal DNA

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ABSTRACT

The present study investigates the genetic identification of *Cinnamomum* species by analyzing the nucleotide sequences of the partial non-coding internal transcribed spacer 2 (ITS2) of ribosomal genes. The genetic diversity of the five *Cinnamomum* species (*Cinnamomum cassia*, *C. zeylanicum*, *C. loureiroi*, *C. osmophloeum* and *C. burmannii*) was studied using polymerase chain reaction (PCR) amplification and a DNA sequencing method to molecularly identify the tested specimens. Phylogenetic trees were constructed using several clustering methods, including unweighted pair-group method with arithmetic average (UPGMA), neighbor-joining (NJ) and maximum evolution (ME). The eleven test samples were clustered into three major groups based on partial ITS2 sequences. The phylogenetic results showed that *C. osmophloeum* and *C. burmanii* are phylogenetically related to each other and that both are more closely related to *C. cassia*. Based on our results, there is enough sequence variation within the sequenced ITS2 regions to identify individual specimens among the five tested *Cinnamomum* species. Therefore, these ITS2 regions could be adopted as a molecular marker for differentiating among *C. cassia*, *C. zeylanicum*, *C. loureiroi*, *C. osmophloem* and *C. burmannii*.

Key words: *Cinnamomum* spp., molecular identification, internal transcribed spacer2 (ITS2), phylogenesis

INTRODUCTION

Cinnamon is the dried bark from a *Cinnamomum* species, such as *C. cassia*, *C. zeylanicum* or related plants. Cinnamon is used as spices and medicinal herb throughout the world[1,2]. The essential oil from cinnamon contains a number of bioactive ingredients, including cinnamaldehyde, coumarin, cinnamyl alcohol and eugenol[3]. Among these bioactive chemicals, cinnamaldehyde is the most abundant and important compound. Several reports have described many bioactive properties of cinnamaldehyde, including its activity as a xanthine oxidase inhibitor[4], an anti-microbial[5], anti-bacterial[6], and a potential anti-diabetic agent[7]. The use of medicinal herbs by the biotechnology industry is growing. The market potential for many health products utilizing cinnamon has been positive.

Cinnamomum belongs to the Lauraceae family and is composed of approximately 110 species of evergreen trees and shrubs[8]. Traditionally, species identification has been based on the morphological or histological characterization of the tree or shrub. However, identification based on morphological characteristics alone is difficult due to the morphological similarities between plants. Recently, sequence comparisons of internal transcribed spacers (ITS) at the DNA level are being widely used as an improved species authentication method[9]. Specifically, the ITS of the nuclear ribosomal 18S-5.8S-26S cistron has been the most popular genomic target for systematic molecular investigations of plants at the species level[10,11]. Sequence variations in the internal transcribed spacer 2 (ITS2) are diverse enough to differentiate between the medicinal *Dendrobium* species[12], along with five other sets of easily confusable Chinese herbal materials[9].

Here we explore the possibility of using the ITS2 region of rDNA to differentiate among five different *Cinnamomum* species. We found that gene-source identification can be performed using ITS2. Hence, the true origin of the plants and quality control can be ensured

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by sequencing ITS2. As a consequence, the sustainable development of the cinnamon bio-industry may be promoted and the competitiveness of the domestic and international market potential for cinnamon products may be strengthened.

MATERIALS AND METHODS

I. *Source of Samples and their Treatment*

Fresh leaves from *Cinnamomum cassia*, *Cinnamomum zeylanicum*, *Cinnamomum loureiroi*, *Cinnamomum osmophloeum* and *Cinnamomum burmannii* were collected from various locations in China, Vietnam and Taiwan. The identification of all of the leaves was authenticated by Dr. Fu-Yuan Lu in the Department of Forestry and Nature Resources at the National Chiayi University. The leaves were dehydrated, aliquoted into air-permeable bags, and stored dryly in sealed containers. Voucher specimens were deposited in the Department of Bioindustry Technology at Da-Yeh University. Sample collection sites and DNA sample codes are listed in Table 1.

II. *DNA Extraction, Polymerase Chain Reaction (PCR) and DNA Sequencing*

Genomic DNA was isolated according to a modified cetyl trimethyl ammonium bromide (CTAB) method with minor modifications[13]. Briefly, 100 mg of the dried leaf material was ground into a fine powder in liquid nitrogen using the mortar and pestle. After the addition of 1 mL of pre-warmed extraction buffer (100 mM TrisHCl, pH 8.0; 20 mM EDTA; 1 M NaCl; 1% CTAB; 1% PVP-40), the mixture was incubated in water bath at 65°C for 20 min with gentle shaking. The sample solution was mixed with an equal volume of chloroform: isoamyl alcohol (24: 1) and centrifuged at 11,000 ×g for 20 min at 4°C. The supernatant was transferred to a new eppendorf tube containing 2 mL of precipitation buffer (50 mM TrisHCl, pH 8.0, 10 mM EDTA, 40 mM NaCl, 1% CTAB), incubated at room temperature for 1 h, and centrifuged at 11,000 ×g for 15 min at 4°C. The supernatant was carefully decanted and the pellet was gently suspended in 350 µL of 1.2 M NaCl with 10 mg/mL RNase A. After incubation at 37°C for 30 min, an extraction with 350 µL of chloroform: isoamyl alcohol (24: 1) was performed, and the aqueous phase was transferred to a new tube. Then, 3 M sodium acetate (one-tenth of the recovered volume) and 95% ethanol (two equal of the recovered volumes) were added to precipitate the DNA. After centrifugation at 12,000 ×g for 20 minutes, the DNA pellet was washed with 1 mL of 70% ethanol, dried and dissolved in the volume of 50 to 100 µL of TE buffer.

The partial ITS2 fragments of five *Cinnamomum* spp. were amplified using the BEL-1/BEL-3 primer set designed by Chiou *et al*[9]. The following specific mixed primer sets were used: Forward primers, BEL-1: 5'-GGDGCGGAKAHTGGCCYCCCGTGC-3', (where D represents A, G or T and K represents G or T, H represents A, C or T and Y represents T or C); BEL-2: 5'-GATGCGGAGATTGGCCCCCCGTGC-3'; Reverse primers, BEL-3: 5'-GACGCTTCTCCAGACTACAAT-3' and BEL-4: 5'-GTTTCTTTTCCTCCGCT-3'. A schematic diagram of the rDNA ITS regions and the designed primers are shown in Figure 1.

Figure 1. Schematic diagram of the rDNA ITS regions and the designed primers. The coding regions of 18S, 5.8S and 26S rDNA are black-boxed. Forward primers (BEL-1 and BEL-2) were derived from a conserved motif close to the 3'-end of the 5.8S rDNA and the reverse primer BEL-3 is complementary to the 5'-terminus of the 26S rDNA. BEL-1, BEL-2 and BEL-3 were used to amplify ITS2. BEL-4 is located 100 bp upstream of BEL-3.

A total volume of 50 µL of prepared solution containing 2 µL of template DNA (40-80 ng), 5 µL of 10x PCR reaction buffer, 3 µL of 25 mM $MgCl_2$, 3 µL of 2.5 mM dNTP, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 0.5 µL (5 units) of *Taq* DNA polymerase (Geneaid Biotech Ltd.; Taipei, Taiwan), 3 µL of DMSO and 31.5 µL of sterile distilled water was used for each PCR solution. The template DNA was denatured at 96°C for 12 min and then subjected to 36 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1.5 min. The final cycle included an extension at 72°C for 10 min. The PCR products were examined with 1.5% agarose gel electrophoresis and purified for sequencing using the ABI PRISM® 377 DNA sequencer (Applied Biosystems Industries; Foster City, CA, USA). The obtained sequences were compiled with the BioEdit software (version 7.0) and confirmed after comparison to the in-house and GenBank databases. The obtained sequences published in this paper were deposited in the GenBank nucleotide sequence databases (GenBank accession GQ255627-GQ255632, GQ255634-GQ255638, Table 1).

III. *Sequence Analysis*

Sequences of the partial ITS2 region were aligned by ClustalW using the BioEdit software (version 7.0). MEGA 3.1[14] software was used to construct the percentage of nucleotide similarity and phylogenetic trees of the five *Cinnamomum* spp. without an outgroup species, based on the UPGMA, the ME and the NJ methods. The default phylogeny test options used to construct UPGMA, ME and NJ phylogenetic trees were: Bootstrap (500 replicates), seed = 22607; Gaps/Missing Data: Complete Deletion; Substitution Model: Nucleotide (kimura 2-parameter); Substitution to include: d: Transitions + Transversions; Pattern among Lineages: Same

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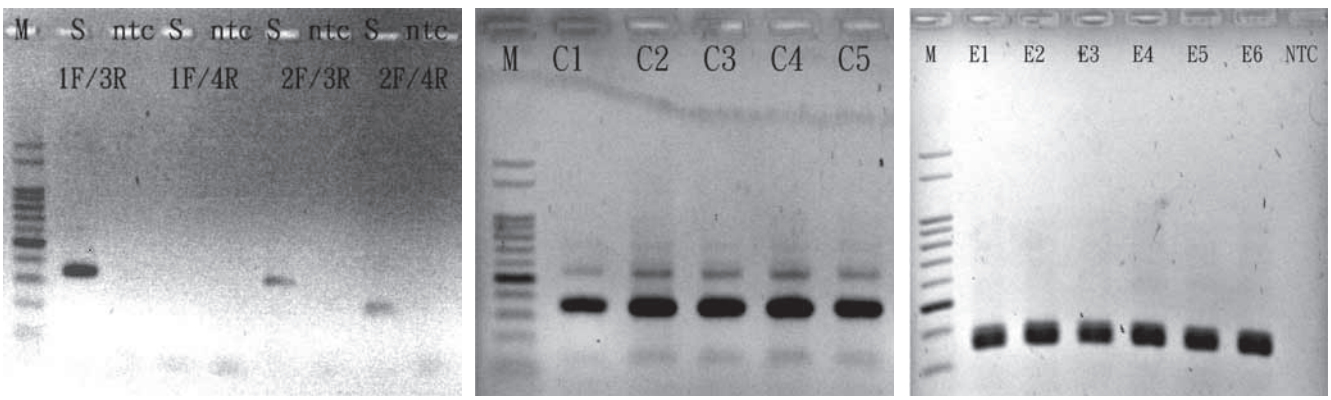
Table 1. Characteristics of the 11 specimens of *Cinnamomum* spp. used in the present study

Code	Organism	Origin	Specimen	GenBank Accession Number
C1	*Cinnamomum cassia*	Fukan, China	CC1	GQ255627
C2	*Cinnamomum cassia*	Fukan, China	CC2	GQ255628
C3	*Cinnamomum cassia*	Guangdong, China	CC3	GQ255629
C4	*Cinnamomum cassia*	Guangdong, China	CC4	GQ255630
C5	*Cinnamomum cassia*	Fukan, China	CC5	GQ255631
E1	*Cinnamomum loureiroi*	Thanh Hoa, Vietnam	CL1	GQ255632
E2	*Cinnamomum zeylanicum*	Colombo, Sri Lanka*	CZ	GQ255634
E3	*Cinnamomum osmophloeum*	Nantou, Taiwan	CO	GQ255635
E4	*Cinnamomum burmannii*	Nantou, Taiwan	CB1	GQ255636
E5	*Cinnamomum burmannii*	Nantou, Taiwan	CB2	GQ255637
E6	*Cinnamomum burmannii*	Nantou, Taiwan	CB3	GQ255638

*Native to Sri Lanka, the plant is cultivated and preserved in Taipei Botanical Garden, Taiwan.



(A) **(B)**

Figure 2. Amplification of ITS2 in *Cinnamomum osmophloeum*. (A) Comparison of the PCR products from *Cinnamomum osmophloeum* with the following primer sets: BEL-1/BEL-3 (Lane S: 1F/3R), BEL-1/BEL-4 (Lane S: 1F/4R), BEL-2/BEL-3 (Lane S: 2F/3R), and BEL-2/BEL-4 (Lane S: 2F/4R). (B) PCR products amplified with the primer set BEL-1/BEL-3 (Lanes C1-C5 and E1-E6). *Cinnamomum* spp. corresponding to each lane is listed in Table 2. The DNA marker (M) is a 100 bp ladder. The 500 bp band has the greatest intensity among the bands. The negative control is a non-template control (NTC).

(Homogeneous); Rate among sites: uniform rates. Additional parameters set up for ME analysis were: CNI (level = 1) with initial tree = NJ, MaxTrees = 1.

RESULTS

I. *Amplification and Sequencing of ITS2*

In the present study, the mixed primer sets (BEL-1, BEL-2, BEL-3 and BEL-4) used were originally designated to amplify the ITS2 region of medicinal plants. Here, we used these mixed primer sets to genetically identify 11 different specimens from five different species of cinnamon (*C. cassia*, *C. zeylanicum*, *C. loureiroi*, *C. osmophloem* and *C. burmannii*.). These primers were developed by Chiou *et al*[9] based on 300 ITSs of commonly used medicinal herbs. These 300 ITS sequences included 120 different species belonging to 52 families. The forward primers, BEL-1 and BEL-2, derived from a conserved motif close to the 3' end of the 5.8S rDNA, and the reverse primer, BEL-3, complementary to the 5' terminus of the 26S rDNA, were used to amplify ITS2 (Figure 1). BEL-4 is located 100 base pairs (bp) upstream of BEL-3. The primer set BEL-1/BEL-3 efficiently amplified the ITS2 region in all of the tested specimens (*C. osmophloem* is shown as an example in Figure 2A). PCR successfully amplified fragments of about 360 bp, including the majority of the ITS2 region and part of the 26S gene (Figure 2B). Direct sequencing

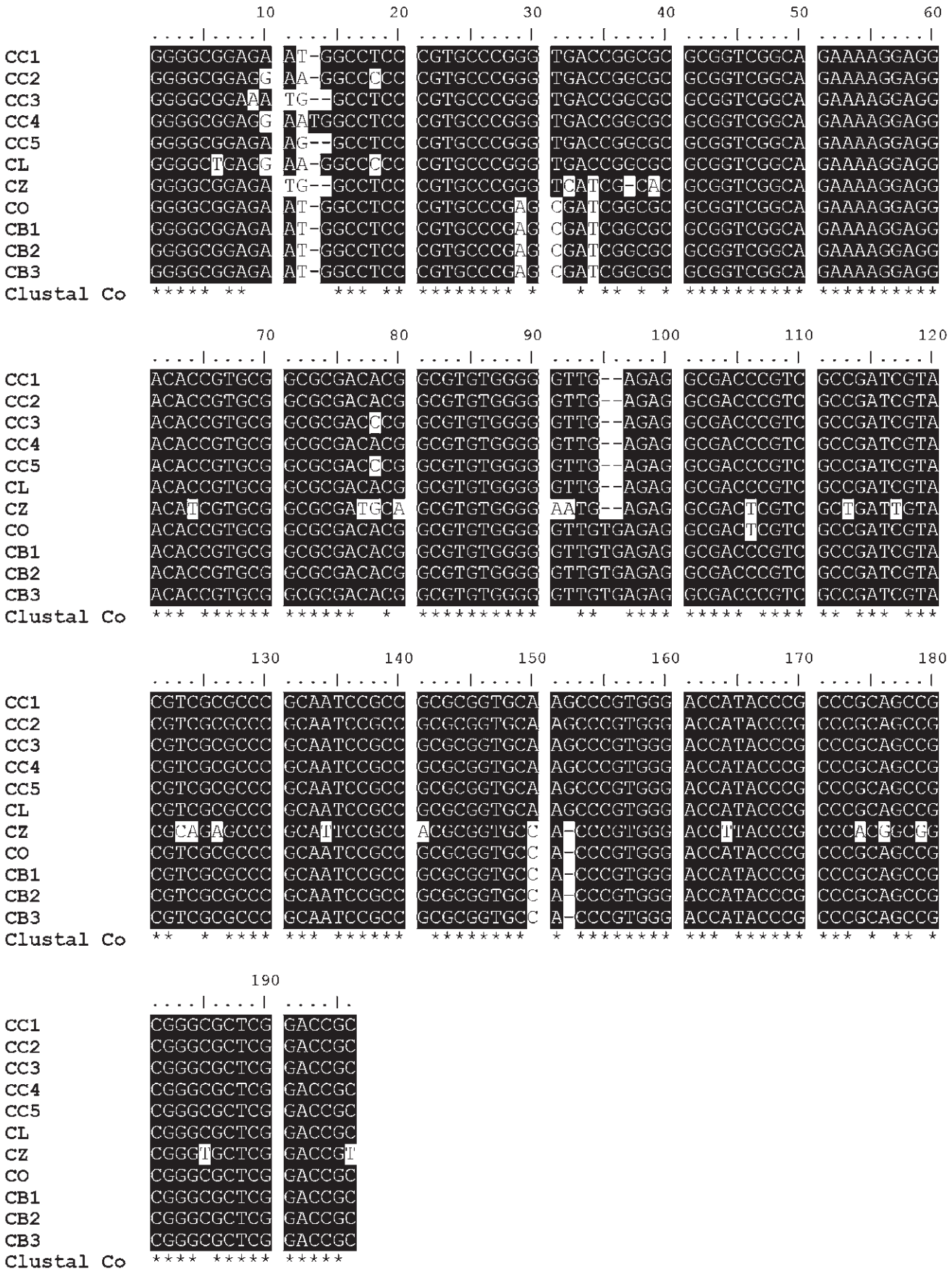


Figure 3. Alignment of the ITS2 sequences of the 11 different *Cinnamomum* spp. specimens. Segments that are conserved between the 11 clones are blacked.

of the 11 specimens of cinnamon was carried out and their sequences were placed in the GenBank database maintained by the National Center for Biotechnology Information (NCBI; Table 1).

II. *Comparison of the ITS2 Sequences*

As shown in Figure 3, intra-specific variation in the ITS2 regions was found to be very low. No difference in the ITS2 regions in the same species was found between the three different specimens of *C. burmannii*. The percent difference among the five specimens of *C. cassia* ranged from 0.6 to 3.2%. Among the 11 specimens of the five different *Cinnamomum* species, the percentage of inter-specific sequence difference ranged from 0.0 to 16.0% (Table 2).

III. *Phylogenetic Analysis*

Based on the sequence and identity differences, a phylogenetic tree was constructed by the UPGMA method for the 11 different specimens of the *Cinnamomum* spp., without an outgroup species (Figure 4A). The 11 specimens clearly clustered into three separated groups. The first group contained all five specimens of *C. cassia*, plus the CL specimen of *C. loureiroi*. The second group possessed four specimens, including all three specimens of *C. burmannii* plus the CO specimen of *C. osmophloeum*. A specimen CZ of *C. zeylanicum* remained in a main branch and differentiated the rest of the specimens. Phylogenetic trees constructed using the NJ and ME tree construction methods for these 11 specimens of *Cinnamomum* species showed the same grouping pattern (Figure 4B and 4C)

DISCUSSION

Several studies have reported the DNA analysis of Lauraceae spp[15-17]. In addition, genetic identification among *Cinnamomum* species have been studied by analyzing the nucleotide sequences of chloroplast DNA from four species (*C. cassia, C. zeylanicum, C. burmannii and C. sieboldii*)[18]. By using nucleotide variation at one site in the *trnL-trnF* intergenic spacer (IGS) and three sites in the *trnL* region, the four *Cinnamomum* species were correctly authenticated. However, there are no reports of genetic identification of *C. osmophloem* or related species based on ITS2 of ribosomal DNA.

Indigenous cinnamon (*C. osmophloeum*) grows in Taiwan's natural hardwood forests at elevations between 400 and 1500 m[19]. Studies on the essential oils extracted from *C. osmophloeum* leaves have revealed its excellent insecticide, anti-bacterial, anti-fungal, anti-inflammatory and anti-oxidant activities[3,4]. As consequences, the market potential for many health products utilizing *C. osmophloeum* has been growing. The *C. burmannii* plant resembles Taiwan's indigenous *C. osmophloeum*. *C. burmannii* and *C. osmophloeum* are often mistaken for one another due to variations in their appearances caused by natural hybridization of the two species. Both species are frequently misidentified during traditional morphological identification[20]. The need for genetic identification of *C. osmophloem* or related species has increased recently.

Genetic identification (or classification) is a method of labeling based on the analysis of DNA polymorphisms. Various types of DNA markers provided by modern molecular biology can reveal differences between species that are not morphologically obvious. These molecular markers can be employed for a broad range

Table 2. Percent difference between the eleven specimens of the *Cinnamomum* species based on the ITS2 region

Seq->	CC1	CC2	CC3	CC4	CC5	CL	CZ	CO	CB1	CB2	CB3
CC1	ID										
CC2	1.6	ID									
CC3	1.6	3.2	ID								
CC4	0.6	2.1	2.1	ID							
CC5	1.6	1.6	2.1	2.1	ID						
CL	2.1	0.6	3.7	2.6	2.1	ID					
CZ	14.5	16.0	14.6	14.9	15.5	16.5	ID				
CO	4.2	5.7	5.7	4.6	5.7	6.2	14.0	ID			
CB1	3.6	5.2	5.2	4.1	5.2	5.7	14.5	0.6	ID		
CB2	3.6	5.2	5.2	4.1	5.2	5.7	14.5	0.6	0	ID	
CB3	3.6	5.2	5.2	4.1	5.2	5.7	14.5	0.6	0	0	ID

Keys: CC1 = *C. cassia* (C1); CC2 = *C. cassia* (C2); CC3 = *C. cassia* (C3); CC4 = *C. cassia* (C4); CC5 = *C. cassia* (C5); CL = *C. loureirori* (E1); CZ = *C. zeylanicum* (E2); CO = *C. osmophloeum* (E3); CB1 = *C. burmannii* (E4); CB2 = *C. burmannii* (E5); CB3 = *C. burmannii* (E6).

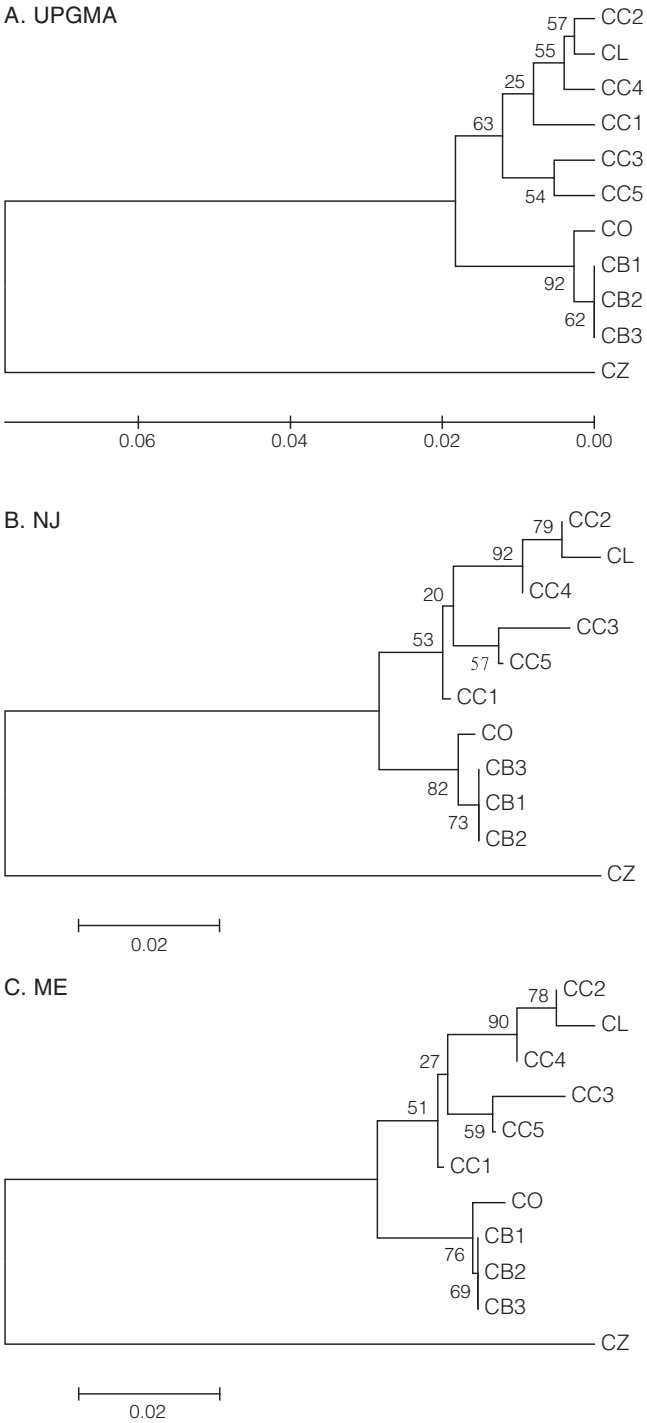


Figure 4. Phylogenetic trees constructed using three different tree construction methods. (A) Phylogenetic tree determined by UPGMA. (B) Phylogenetic tree determined by NJ. (C) Phylogenetic tree determined by ME.

of purposes, including species identification[21], phylogenetic studies[22], the study of system evolution[23] and geological phylogenetics[24]. Most DNA mutations are silent mutations and do not affect the phenotype. Silent mutations allow the changes in the DNA to persist and be used to reliably classify and identify the plant species after direct DNA sequencing.

ITSs are useful markers for inter-specific differentiation[25]. Sequencing of the ITS region is widely applied in plant phylogenetic inference and in the authentication of Chinese medicine from its adulterants[9,10]. Two ITS regions are located between the repeating array of nuclear 18S, 5.8S and 26S ribosomal RNA genes, a locus that has 100-200 copies per genome[26]. Within individuals and species, rRNA genes are highly homologous as a consequence of homologous recombination and gene conversion[23]. Chiou *et al*.[9] successfully established a database of ITS sequences from over 300 species of commonly used Chinese herbal materials. Furthermore, a primer set designed by Chiou *et al*.[9] led to an accurate PCR product of the specific ITS2 region correlating with DNA extracted from 55 processed medicinal herbs belonging to 48 families. Moreover, Chiou *et al*.[9] selectively amplified ITS2 and authenticated five sets of easily confused Chinese herbal materials. These designed primers proved to be suitable for a broad application in the authentication of herbal materials.

Our molecular results show that the average percent difference in the intra-species sequence was 1.86% for *C. cassia*. Additionally, the ITS2 region was identical for all three tested intra-species of *C. burmannii*. Our results also show that the percent difference in the inter-species sequence among the five *Cinnamomum* species ranged from 0.6 to 14.5% (with an average of 6.9%), which is much larger than the intra-species variation of 0% for *C. burmannii* and of 1.86% for *C. cassia*. The average percentage of inter-species difference in the ITS2 regions of *Dendrobium* species is 12.4%[12], while the average difference among the five *Cinnamomum* species is about 6.9%. Therefore, the ITS2 region of the *Cinnamomum* species may be adopted as a molecular marker for the accurate identification of these species. The use of traditional identification methods, such as comparative morphology, can sometimes result in the mislabeling and subsequent misuse of these species. Using sequence-specific primers that amplify rDNA ITS2 sequences, molecular markers could be used as a more reliable species identification method.

CONCLUSIONS

Based on our results, there is enough sequence variation within the ITS2 regions of the five tested *Cinnamomum* spp. to identify individual specimens. Therefore, the ITS2 regions could be adopted as molecular markers to differentiate among *C. cassia*, *C. zeylanicum*, *C. loureiroi*, *C. osmophloem* and *C. burmannii*.

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REFERENCES

1. Lai, P. K. and Roy, J. 2004. Antimicrobial and chemopreventive properties of herbs and spices. Curr. Med. Chem. 11: 1451-1460.
2. Mishra, A., Bhatti, R., Singh, A. and Singh, I. M. P. 2009. Ameliorative effect of the cinnamon oil from *Cinnamomum zeylanicum* upon early stage diabetic nephropathy. Planta Med. 76: 412-417.
3. Cheng, S. S., Liu, J. Y., Hsui, Y. R. and Chang, S. T. 2006. Chemical polymorphism and antifungal activity of essential oils from leaves of different provenances of indigenous cinnamon (*Cinnamomum osmophloeum*). Bioresour. Technol. 97: 306-312.
4. Wang, S. Y., Yang, C. W., Liao, J. W., Zhen, W. W., Chu, F. H. and Chang, S. T. 2008. Essential oil from leaves of *Cinnamomum osmophloeum* acts as a xanthine oxidase inhibitor and reduces the serum uric acid levels in oxonate-induced mice. Phytomedicine 15: 940-945.
5. Ooi, L. S., Li, Y., Kam, S. L., Wang, H., Wong, E. Y. and Ooi, V. E. 2006. Antimicrobial activities of cinnamon oil and cinnamaldehyde from the Chinese medicinal herb *Cinnamomum cassia* Blume. Am. J. Chin. Med. 34: 511-522.
6. Shan, B., Cai, Y. Z., Brooks, J. D. and Corke, H. 2007. Antibacterial properties and major bioactive components of cinnamon stick (*Cinnamomum burmannii*): activity against foodborne pathogenic bacteria. J. Agric. Food Chem. 55: 5484-5490.
7. Subash-Babu, P., Prabuseenivasan, S. and Ignacimuthu, S. 2007. Cinnamaldehyde--a potential antidiabetic agent. Phytomedicine 14: 15-22.
8. Purseglove, J. W. 1969. Lauraceae. In"Tropical Crops: Dicotyledons 2". 2nd ed. pp. 187-192. Purseglove, J. W. ed. Longmans Green and Co. ltd. London, U.K.
9. Chiou, S. J., Yen, J. H., Fang, C. L., Chen, H. L. and Lin, T. Y. 2007. Authentication of medicinal herbs using PCR-amplified ITS2 with specific primers. Planta Med. 73: 1421-1426.
10. Alvarez, I. and Wendel, J. F. 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phylogenet. Evol. 29: 417-434.
11. Beltrame-Botelho, I. T., Gaspar-Silva, D., Steindel, M., Dávila, A. M. and Grisard, E. C. 2005. Internal transcribed spacers (ITS) of *Trypanosoma rangeli* ribosomal DNA (rDNA): a useful marker for inter-specific differentiation. Infect. Genet. Evol. 5: 17-28.
12. Lau, D. T., Shaw, P. C., Wang, J. and But, P. P. 2001. Authentication of medicinal *Dendrobium* species by the internal transcribed spacer of ribosomal DNA. Planta Med. 67: 456-460.
13. Cubero, O. F., Crespo, A., Fatehi, J. and Bridge, P. D. 1999. DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. Plant Syst. Evol. 216: 243-249.
14. Kumar, S., Tamura, K. and Nei, M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinformatics 5: 150-163.
15. Renner, S. S. 1999. Circumscription and phylogeny of the Laurales: evidence from molecular and morphological data. Am. J. Bot. 86: 1301-1315.
16. Rohwer, J. G. 2000. Toward a phylogenetic classification of the Lauraceae: evidence from *matK* sequences. Systemat. Bot. 25: 60-71.
17. Savolainen, V., Chase, M. W., Morton, C. M., Hoot, S. B., Soltis, D. E., Bayer, C., Fay, M. F., Bruijn, A., Sullivan, S. and Qiu, Y. L. 2000. Phylogenetics of flowering plants based upon a combined analysis of plastid *atpB* and *rbcL* gene sequences. Syst. Biol. 49: 306-362.
18. Kojoma, M., Kurihara, K., Yamada, K., Sekita, S., Satake, M. and Iida, O. 2002. Genetic identification of cinnamon (*Cinnamomum* spp.) based on the *trnL-trnF* chloroplast DNA. Planta Med. 68: 94-96.
19. Liu, Y. C., Lu, F. Y. and Ou, C. H. 1988. Trees of Taiwan. Monographic Publication No. 7. College of Agriculture, National Chung-Hshing University, Taichung, Taiwan, R.OYin, H. W., Chen, C. F. and Lu, S. Y. 2007. Morphological features and essential oil characteristics of *Cinnamomum burmannii* and *C. osmophloeum*. Q. J. Chin. For. 40: 535-548. (Article in Chinese)
20. Viñas, J. and Tudela, S. 2009. A validated methodology for genetic identification of tuna species (genus *Thunnus*). PLoS One 4: e7606.
21. Schönhuth, S. and Mayden, R. L. 2009. Phylogenetic relationships in the genus *Cyprinella* (*Actinopterygii: Cyprinidae*) based on mitochondrial and nuclear gene sequences. Mol. Phylogenet. Evol. 55: 77-98.
22. Hillis, D. M. and Dixon, M. T. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Q. Rev. Biol. 66: 411-453.
23. Goka, K., Yokoyama, J., Une, Y., Kuroki, T., Suzuki, K., Nakahara, M., Kobayashi, A., Inaba, S., Mizutani, T. and Hyatt, A. D. 2009. Amphibian chytridiomycosis in Japan: distribution, haplotypes and possible route of entry into Japan. Mol. Ecol. 18: 4757-4774.
24. Beltrame-Botelho, I. T., Gaspar-Silva, D., Steindel, M., Dávila, A. M. and Grisard, E. C. 2005. Internal transcribed spacers (ITS) of *Trypanosoma rangeli* ribosomal DNA (rDNA): a useful marker for inter-specific differentiation. Infect. Genet. Evol. 5: 17-28.
25. Hernandez, R., Martinez-Calvillo, S., Hernandez-Rivas, R. and Gomez, E. 1993. *Trypanosoma cruzi* ribosomal RNA genes: a review. Biol. Res. 26: 109-114.