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DNA Barcoding *Cinnamomum osmophloeum* Kaneh. Based on the Partial Non-Coding ITS2 Region of Ribosomal Genes

SHIH-CHIEH LEE[1]*, SHU-JIAU CHIOU[2,3], JUI-HUNG YEN[2], TSAI-YUN LIN[3], KUN-TING HSIEH[4] AND JENG-CHUAN YANG[5]

[1.] *Department of Bioindustry Technology, Da-Yeh University, Changhua 51591, Taiwan, R.O.C.*

[2.] *Biomedical Engineering Research Laboratories, Industrial Technology Research Institute, Hsinchu 31040, Taiwan, R.O.C.*

[3.] *Department of Life Science and Institute of Bioinformatics and Structural Biology, National Tsing Hua University, Hsinchu 30013, Taiwan, R.O.C.*

[4.] *Department of Molecular Biotechnology, Da-Yeh University, Changhua 51591, Taiwan, R.O.C.*

[5.] *Department of Forestry and Nature Conservation, Chinese Culture University, Taipei 11114, Taiwan, R.O.C.*

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ABSTRACT

This paper investigated the DNA barcoding of indigenous cinnamon. The essential oil composition of leaves and the partial non-coding internal transcribed spacer (ITS2) region of ribosomal genes from indigenous cinnamon plants (*Cinnamomum osmophloeum* Kaneh.) were investigated. Leaf essential oils from seven geographical strains of *C. osmophloeum* Kaneh. were obtained by hydrodistillation and characterized by gas chromatography-mass spectrometry. Six chemotypes, including cinnamaldehyde, cinnamaldehyde/cinnamyl acetate, cinnamyl acetate, linalool, camphor and mixed, were identified. Seven representative geographical strains were further used to study their genetic diversity based on the partial ITS2 region using PCR amplification and DNA sequencing to evaluate the region's use in the application of DNA barcoding *C. osmophloeum* Kaneh. A phylogenetic tree was constructed using UPGMA clustering method. Our results indicate that the partial ITS2 nucleotide sequences for all seven geographical strains are identical and not correlated with essential oil composition. The partial ITS2 sequences is sufficient for barcoding *C. osmophloeum* Kaneh., while that additional genes will need to be analyzed to identify samples of various chemotypes. We believe that this partial ITS2 locus serves as a good starting point for large-scale testing of DNA barcoding of other plant species across Taiwan.

Key words: *Cinnamomum osmophloeum* Kaneh., chemotype, internal transcribed spacer, barcode

INTRODUCTION

Cinnamomum is a genus belonging to the Laurel family, Lauraceae. Indigenous cinnamon (*Cinnamomum osmophloeum* Kaneh.) grows in Taiwan's natural hardwood forests at elevations between 400 and 1500 m[(1)]. Many plants within this genus have been used in folk medicine for their interesting biological activities, including anti-diabetic, anti-inflammatory, astringent and diuretic effects, as well as their ability to heal intestinal infections[(2)].

Studies on the essential oils extracted from *C. osmophloeum* Kaneh. leaves have demonstrated excellent insecticidal[(3-5)], anti-bacterial[(6,7)], anti-fungal[(8,9)], anti-inflammatory[(10-15)] and anti-oxidant[(16)] activities. Based on the chemical composition of the leaf essential oils and cluster analyses of their relative contents, sixteen different geographical strains of indigenous cinnamon have been classified into six chemotypes: cinnamaldehyde, cinnamaldehyde/cinnamyl acetate, cinnamyl acetate, linalool, camphor and mixed[(17)]. Phytochemical analyses and biological screenings have demonstrated that the cinnamaldehyde chemotype presents the highest anti-fungal and insecticidal activities among all *C. osmophloeum* Kaneh. chemotypes[(8)]. Cinnamaldehyde has been reported as a potential anti-diabetic agent[(18)], and is also valued for its chemoprotective[(19)] and anti-inflammatory effects[(20,21)].

The internal transcribed spacer (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[(22-25)]. Comparison

* Author for correspondence. Tel: +886-4-8511888 ext. 4262; Fax: +886-4-7236219; E-mail: slee@mail.dyu.edu.tw

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studies of homologous ITS sequences are also widely used as an improved method to identify medicinal herbs using DNA[26-28]. Species, and even varieties within species, can be distinguished by their ITS region nucleotide sequences[29-31]. Other than the ITS method, cinnamon species had also been identified genetically by analyzing the nucleotide sequences of chloroplast DNA from four species (*Cinnamomum cassia*, *C. zeylanicum*, *C. burmannii* and *C. sieboldii*)[32].

DNA barcoding is a method for identifying species using short orthologous DNA sequences. This strategy has facilitated biodiversity studies, identified juveniles, associated sexes, and enhanced forensic analysis[33-35]. Because of its high degree of accuracy, a great deal of effort has been made in developing genome-based methods to identify and barcode plants, particularly those with medicinal applications[36-40].

The need for barcoding in plants has increased recently. The use of ITS sequences is generally accepted for the molecular analysis of plants, but its primary purpose is to identify species, rather than to discriminate/identify specimens/varieties. So we sought to identify a nucleotide sequence for DNA barcoding, based on the ITS2 region of the ribosomal RNA genes, which can be properly used for distinct chemotypes of *C. osmophloeum* Kaneh. We believe that the successful acquisition of this partial ITS2 locus may serve as a good starting point for large-scale testing of DNA barcoding across a large amount of plant species in Taiwan.

MATERIALS AND METHODS

I. *Isolation of Essential Oils*

Fresh leaves (old and young leaves were not used) from seven *C. osmophloeum* Kaneh. geographical strains (B1, D4, D17, G2, LL, P3, T1) were collected from the Lien Hua-Chin Research Center located in Nantou County, in central Taiwan (Table 1). Geographical strains of *C. osmophloeum* Kaneh. were collected (and identified) by Dr. Ta-Wei Hu and his colleagues from various locations in Taiwan and were planted in Lien Hua-Chin Research Center in 1992[41]. Tested materials were also planted in the same location in 1992, so all the geographical strains were grown under similar conditions and the environmental stress was minimized. The leaves were collected in the fall of 2007. The voucher specimens were deposited at the Department of Bioindustry Technology, Da-Yeh University. The leaves were kept in a -20ºC freezer after harvest from the field and were analyzed as soon as possible thereafter. Triplicate samples (150 g each) were subjected to hydrodistillation in a Clevenger-type apparatus for 6 h[7], after which the oil contents were identified. Leaf essential oils were stored in airtight containers prior to analysis by gas chromatography-mass spectrometry (GC-MS).

Table 1. Seven geographical strains of *C. osmophloeum* Kaneh. used in the present study

Prov. Code	Strain or accession name
B1	Bailu 1
D4	Taidiansiduan 4
D17	Taidiansiduan 17
G2	Jiabaotai 2
LL	Lilong
P3	Pulizhuzilai 3
T1	Cengwen 1

II. *GC/MS Analysis*

A QP2010 gas chromatograph/mass spectrometer (Shimadzu, Japan) was used with the following settings: RTx-5 capillary column, 30 m × 0.25 mm × 0.25 μm (Restek Corp., USA); helium carrier gas flow, 10.0 mL/min 1:10 split ratio; injection temperature, 200°C; interface temperature, 200°C; ion source temperature, 200°C; ion mode, electron impact ionization (70 eV)/selected ion monitoring mode; oven temperature program, 80°C for 1 min, 4°C/min to 200°C and held for 14 min, then 10°C /min to 280°C and held for 2 min. Diluted samples (1.0 μL, 1/100, v/v in methanol) were injected manually in the splitless mode. The components of *C. osmophloeum* Kaneh. leaf oils were identified by comparison with standard peaks and mass spectra in the NIST 2005 library. The quantities of the compounds were determined by integrating the area under the peaks of the spectra. Selected compounds, which are components of essential oils, were quantified using a linear calibration curve with standard solutions of each individual standard compound dissolved in methanol at concentrations ranging between 10 and 500 ng/mL with 50 ng/mL of that selected standard compound as an internal standard.

III. *Essential Oil Components*

The following compounds, which are components of essential oils, were purchased from Acros Organics (Geel, Belgium): benzaldehyde, linalool, α-terpineol, neral, geraniol, eugenol, cinnamyl alcohol, and cinnamaldehyde. The cinnamaldehyde congeners, including cinnamic acid, cinnamyl acetate, 4-hydroxybenzaldehyde and 3-phenylpropionaldehyde, were also obtained from Acros Organics (Geel, Belgium).

IV. *Cluster Analysis*

Cluster analysis was performed with MVSP software (version 3.1) to identify a relatively homogeneous group of seven geographical strains (B1, D4, D17, G2,

LL, P3 and T1) of *C. osmophloeum* Kaneh. based on the percent composition of their essential oils. Euclidean distance was selected as a measure of similarity and the unweighted pair-group method with arithmetic average (UPGMA) was used to define clusters.

V. *Molecular Methods*

DNA isolation, polymerase chain reactions (PCR) and DNA sequencing were performed according to the procedures outlined in Chiou *et al.*[26]. Dried plant leaf material (100 mg) was used for genomic DNA extraction. The specific mixed forward primer BEL-1 (5′-GGDGCG-GAKAHTGGCCYCCCGTGC-3′ where D represents A, G or T; K represents G or T; H represents A, C or T; and Y represents T or C) was designed based on the occurrence of A, T, G and C at each position in the conserved region of ITS2 of the 120 representative species. The reverse primer BEL-3 (5′-GACGCTTCTCCAGACTA-CAAT-3′), located approximately 160 nucleotides from the 5′ terminus of the 26S rRNA sequence, is specific to plants (Figure 1). The ITS2 and 26S fragments (both are partial) of seven representative geographical strains of *C. osmophloeum* Kaneh. were amplified using the primer set BEL1/BEL3. Each 50 μL PCR solution contained 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) *Taq* DNA polymerase (Geneaid Biotech Ltd.; Taipei, Taiwan), 3 μL DMSO and 31.5 μL sterile distilled water. The template DNA was denatured at 94°C for 10 min and then subjected to 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec. The final cycle included an extension period of 10 min at 72°C. The PCR products were examined on a 1.5% agarose gel and purified for sequencing. Single strand of the purified PCR product was subjected to direct 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.

VI. *Phylogenetic Analysis Based on rRNA Sequences*

Sequences of the partial ITS2 region were aligned by ClustalW using the BioEdit software (version 7.0). MEGA 3.1[42] software was used to construct phylogenetic trees of the seven representative geographical strains without any outgroup, based on the UPGMA method. The default phylogeny test options used to construct UPGMA 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 (Homogeneous); Rate among sites: uniform rates.

RESULTS AND DISCUSSION

I. *Chemical Composition of Essential Oils*

The compositions of the essential oils from the seven samples are reported in Table 2. A total of 34 compounds were identified in the seven leaf essential oils, constituting 86.71-100.01% of the oils. Unidentified components are compounds that belong neither to the known standards, which are listed in the materials and methods section, nor to the known chemicals in the NIST 2005 GC-MS library. The major components of leaf essential oils are linalool (89.14%), trans-cinnamaldehyde (73.27%), cinnamyl acetate (70.80%) and camphor (55.94%), for geographical strains LL, D4, G2 and T1, respectively. The leaf essential oil from strain D17 that account for 99.17% of the oil's composition, with trans-cinnamaldehyde (48.25%) and cinnamyl acetate (45.89%) being the main components. Only minute amounts of trans-cinnamaldehyde (1.80%) were found in the leaf essential oil from strain B1. No trans-cinnamaldehyde was identified in the leaf essential oils from strains P3 and T1.

II. *Cluster Analysis*

Cluster analysis of the identified compounds allowed for classification of the leaf oils into six main groups, at a Euclidean distance of 40, based on their main components (Figure 2, Table 2). The groups consisted of the following: geographical strain D4; geographical strains G2 and D17; geographical strain B1; geographical strain LL; geographical strain T1; and geographical strain P3. On the basis of the classification established by Cheng *et al.*[8] and the results obtained from cluster analysis in this study, we classified the leaf essential oil of geographical strain

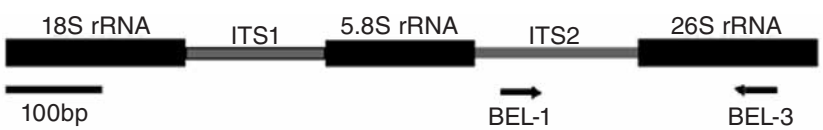

Figure 1. Schematic diagram of the rRNA, ITS regions and designed primers.
The coding regions of 18S, 5.8S and 26S rRNA are indicated by black boxes. Forward primers BEL-1derived from a conserved motif close to the 3′ end of 5.8S rDNA and reverse primer BEL-3 complementary to the 5′ of 26S rDNA were used to amplify partial ITS2 and 26S regions.

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Table 2. Chemical compounds in leaf essential oils from seven strains of *C. osmophloeum* Kaneh. (%)

Compounds (RT[a])	[1]B1	[2]D4	[3]D17	[3]G2	[4]LL	[5]P3	[6]T1
Camphene (3.97)	-[b]	0.19	-	-	-	-	0.79
Benzaldehyde (4.14)	-	0.77	0.49	0.29	-	-	-
β-Phellandrene (4.42)	-	-	-	-	-	-	0.45
β-Pinene (4.52)	-	0.14	-	-	-	-	0.75
Limonene (5.34)	-	-	-	-	-	-	2.60
Salicylaldehyde (5.72)	-	-	-	-	-	-	0.43
Linalool (6.85)	1.07	-	-	-	**89.14**	-	-
Benzyl alcohol (8.20)	-	-	-	0.13	-	-	-
Camphor (8.23)	-	-	-	-	-	-	**55.94**
Benzenepropanal (8.64)	-	0.84	0.54	0.31	-	-	-
2-Methylbenzofuran (9.06)	24.32	0.39	0.27	0.25	2.10	16.90	7.24
α-Terpineol (9.43)	-	-	-	-	0.89	-	0.80
p-Allylanisole (9.62)	-	0.24	0.25	0.21	0.41	0.98	-
Geraniol(10.20)	-	-	-	-	0.33	-	-
cis-Cinnamaldehyde (11.27)	-	0.49	0.37	0.13	-	-	-
***trans*-Cinnamaldehyde (11.77)**	**1.80**	**73.27**	**48.25**	**26.85**	**2.55**	**-**	**-**
Bornyl acetate (12.21)	-	1.43	0.61	0.32	-	3.50	18.21
trans-cinnamyl alcohol (12.80)	-	-	0.12	-	-	-	-
Eugenol (14.39)	-	0.54	0.31	-	-	-	-
α-Cubebene (14.99)	-	0.20	-	-	-	4.66	0.47
Geranyl acetate (15.10)	3.00	0.12	-	-	-	3.93	0.74
β-Caryophyllene (16.36)	7.05	2.91	0.46	0.34	2.70	15.03	2.83
Cinnamyl acetate (17.03)	**48.36**	17.18	**45.89**	**70.80**	0.31	3.31	-
α-Caryophyllene (17.38)	0.84	0.49	-	-	-	2.13	0.53
γ-Muurolene (18.20)	1.40	-	-	-	-	2.63	-
α-Muurolene (18.73)	-	-	-	-	-	1.97	-
γ-Elemene (18.67)	2.76	0.08	-	0.13	0.58	-	-
Isoledene (19.41)	3.61	0.22	0.21	0.25	0.64	13.56	1.39
Caryophyllene oxide (21.22)	0.66	0.17	-	-	-	-	-
Guaiol (21.58)	-	-	-	-	-	5.16	-
T-Cadinol (22.81)	1.65	-	-	-	-	6.55	0.40
α-Cadinol (23.19)	2.76	-	-	-	-	6.40	-
Rimuen (30.37)	-	-	0.76	-	-	-	-
Verticiol (32.19)	-	-	0.94	-	-	-	-
Identified compounds (%)	**99.28**	**99.67**	**99.17**	**100.01**	**99.65**	86.71	93.57

Chemotype: [1]cinnamyl acetate; [2]cinnamaldehyde; [3]cinnamaldehyde/cinnamylacetate; [4]linalool; [5]mixed; [6]camphor.
[a] RT: Retention time (min); [b] "-": Not detected.

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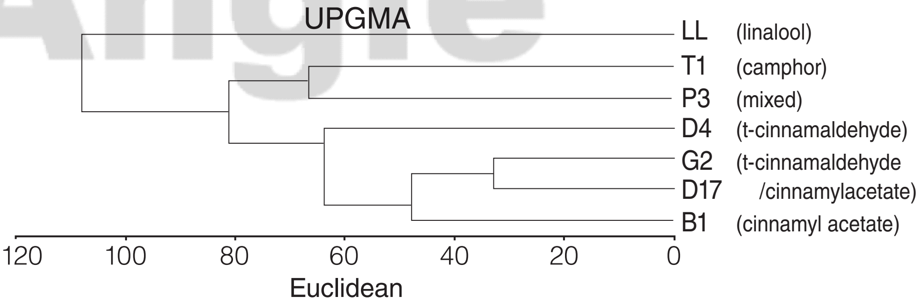

Figure 2. Dendrogram obtained by cluster analysis of the percentage composition of essential oils from seven geographical strains of *C. osmophloeum* Kaneh.

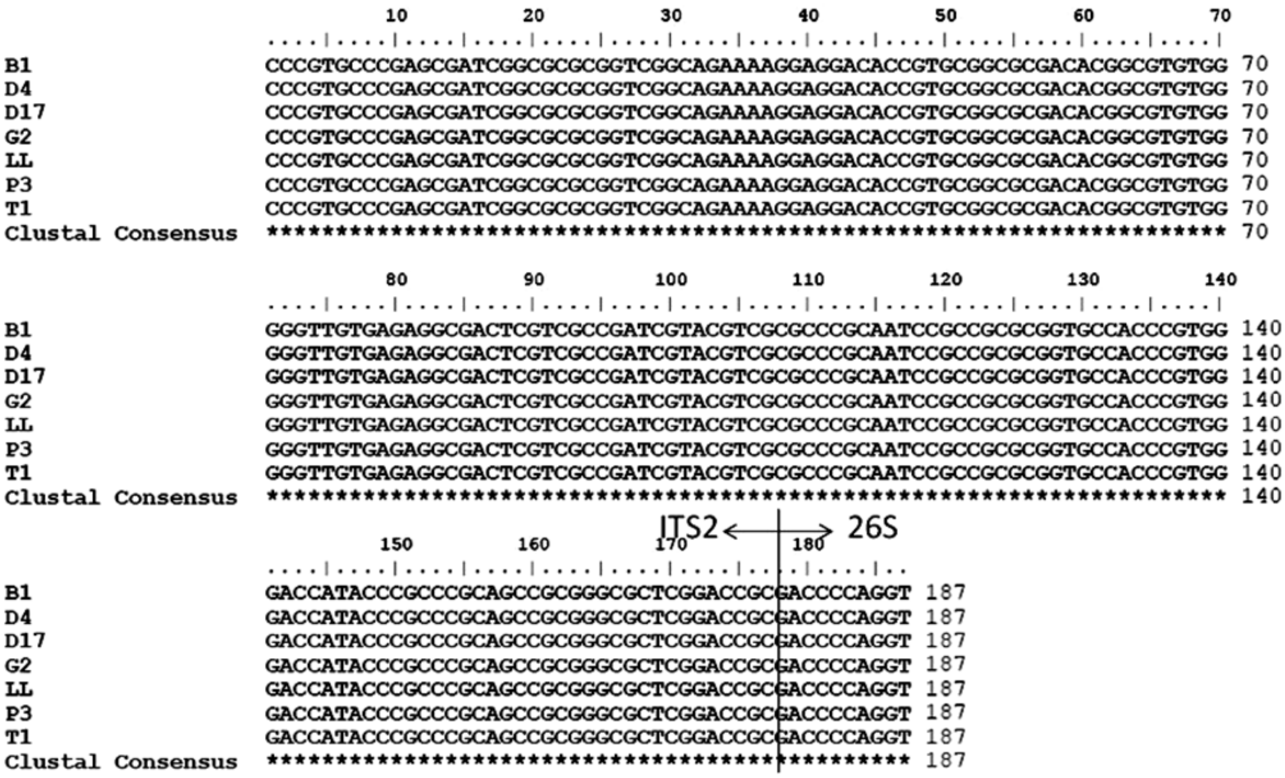

Figure 3. Multiple alignment of the partial ITS2 and 26S sequences of the seven representative geographical strains, including Bailu 1 (B1), Taidiansiduan 4 (D4), Taidiansiduan 17 (D17), Jiabaotai 2 (G2), Lilong (LL) and Pulizhuzilai 3 (P3), Cengwen 1 (T1), of *C. osmophloeum* Kaneh. used in the study.

D4 as cinnamaldehyde type, those of strain G2 and D17 as cinnamaldehyde/cinnamyl acetate type, that of strain B1 as cinnamyl acetate type, that of strain LL as linalool type and that of strain T1 as camphor type. However, the essential oil from strain P3 was classified as a mixed type because of the lack of a dominant compound. Seven representative geographical strains were used as model materials (based on their chemotype) to further investigate the genetic diversity of the ITS2 region.

III. *DNA Sequencing and Phylogenetic Analysis*

Partial ITS2 and partial 26S fragments of seven representative geographical strains of *C. osmophloeum* Kaneh. were amplified and sequenced. Only the portion of sequences directly after the primer BEL-1 in the ITS2 region and 10 conserved nucleotides in the beginning of 5′ end in the 26S ribosomal gene region were used for sequence alignment by ClustalW to observe individual differences. The borders of the ITS2 and 26S regions are shown in Figure 3. A total of 187 positions were observed after ClustalW multiple sequence alignment. The pairwise similarity is 100% among the partial ITS2 sequences of test samples from different geographical strains based on the calculation using the BioEdit program (data not shown here). This analysis revealed

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complete sequence homology among all seven tested chemotypes of the geographical strains. The phylogenetic trees for the test samples in this study were clustered into only one group in the UPGMA dendrogram based on the partial ITS2 sequence data (data not shown here). Interestingly, they represent six different chemotypes of *C. osmophloeum* Kaneh. with highly divergent chemical composition of leaf essential oils, as determined by our GC-MS analysis.

Phytochemical variation and its correlation with genetic markers in other plant species, both within and between geographical species, have been investigated. For example, the DNA sequence of the 5S rRNA intergenic spacer domain was used as a species identifier and differences in both intra- and interspecies phytochemical fingerprints were established by HPLC[43-45]. Whole-genome Random Amplification of Polymorphic DNA (RAPD) or Arbitrarily Primed PCR (AP-PCR) patterns exhibited more variation at the species level than at the level of single DNA region sequences. For example, *Astragalus membranaceus* samples collected from different geographical regions in China exhibited identical ITS1 sequences but different AP-PCR fingerprints[43]. Dong and colleagues determined the DNA sequences of the 5S rRNA spacer, the ITS and the 18S rRNA coding region in 10 different taxa of *Astragalus*[46]. They used multiple bioinformatic tools to construct phylogenetic trees, with each genetic region as an input. In fact, sequence data from a single gene may not be sufficient for barcoding purposes in plants because multiple closely related species have been found to possess identical sequences at some loci. Consequently, the consensus view is that the unequivocal identification and barcoding of all plant species will require sequence data from more than one locus.

Chinese medicines need to be prepared from the correct plant species. Chiou *et al.*[26] have successfully established a database of ITS sequences from over 300 species of commonly used Chinese herbal materials. Furthermore, a primer set that was designed by Chiou *et al.*[26] led to an accurate PCR product of the specific ITS2 region, which was correlated with DNA extracted from 55 processed medicinal herbs belonging to 48 families. These designed primers were proven to be suitable for broad application in the authentication of herbal materials.

The use of genome-based methods to identify medicinal plants in the context of plant phylogenetic studies, and a general effort aimed at barcoding all plants, has received great attention recently[33,40]. The generation of molecular "barcodes" of medicinal plants and deposition of sequence data in publicly accessible databases is worth the concerted effort of the medicinal plant research community. In plants, the plastid locus *rbc*L is commonly sequenced for phylogenetic purposes and also has been suggested as a candidate for plant barcoding. However, no region of the plastid genome has been found to have the high level of variation seen in most animal CO1 barcodes. Kress *et al.* have proposed three criteria that must be met in order for a genetic locus to be useful for plant DNA barcoding: (i) significant species level genetic variability and divergence, (ii) an appropriately short sequence length so as to facilitate DNA extraction and amplification, and (iii) the presence of conserved flanking sites for developing universal primers[39]. We therefore suggest that ITS2 is an appropriate genetic locus for DNA barcoding purposes with respect to the three barcoding criteria: amplification success, sequence length, and sequence divergence. The 26S locus provides a baseline against which to compare other genes and intergenic spacers in our directed search for sequences to use in plant DNA barcoding. The primer set BEL-1/BEL-3 was used in this study to guarantee the successful amplification of the partial ITS2 region of indigenous cinnamon. Because short sequence length is an important criterion for barcoding, the conserved nucleotide sequences in ITS2, in combination with ITS2's relatively short length, is a significant advantage. We believe that the ITS2 locus may serves as a good starting point for the testing of DNA barcoding in many angiosperms across Taiwan.

CONCLUSIONS

The ITS2 sequence can serve to barcode specimens from different strains of *Cinnamomum osmophloeum* Kaneh., while additional genes may need to be analyzed to identify samples of various chemotypes.

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