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ORIGINAL ARTICLE

Rapid Identification of the Indigenous Medicinal Crop *Cinnamomum osmophloeum* from Various Adulterant *Cinnamomum* Species by DNA Polymorphism Analysis

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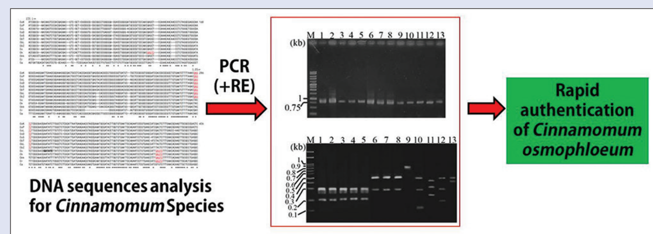
ABSTRACT

Background: *Cinnamomum osmophloeum* (*Co*), a member of the *Lauraceae*, is an indigenous medicinal crop in Taiwan, and it contains higher cinnamaldehyde in essential oil than do other *Cinnamomum* species. Among these species, *Cinnamomum burmannii* (*Cb*) is frequently adulterated as *Co* because of their similar morphological characteristics or features. **Objective:** The purpose of this study was to develop a DNA-based molecular method for rapid authentication of the indigenous *Co* and prevention of its adulteration. **Materials and Methods:** The internal transcribed spacer (ITS) regions of nuclear ribosomal DNA from various *Cinnamomum* species were amplified by polymerase chain reaction (PCR), and these obtained sequences were used for sequence analysis. Based on the sequence variants among various *Cinnamomum* species, restriction fragment length polymorphism (RFLP) was used to differentiate these *Cinnamomum* plants. **Results:** Two restriction endonucleases, *Myl*I and *Eco*RV, were specifically used to digest the PCR-amplified ITS DNA from seven *Cinnamomum* species. The PCR-RFLP results demonstrated that the different restriction patterns that were produced by these two restriction enzymes clearly distinguished *Co* from *Cb* and five other *Cinnamomum* species simultaneously. **Conclusion:** The PCR-RFLP analysis developed in this study provides an alternative method for rapidly identifying *Cinnamomum* plants at the species level using DNA polymorphisms.

Key words: *Cinnamomum burmannii*, *Cinnamomum osmophloeum*, internal transcribed spacers, polymerase chain reaction, restriction fragment length polymorphism

SUMMARY

- Polymerase chain reaction-restriction fragment length polymorphism method described in the present study enabled us to rapidly and conveniently identify *Cinnamomum osmophloeum (Co)* from various *Cinnamomum* species. It may be useful and easy to standardize this method, which can be applied practically for *Cinnamomum* identification. Moreover, in the future, it might be possible to apply this approach for the investigation of the population diversity and structure of *Co* and other *Cinnamomum* species that are indigenous to Taiwan.



Abbreviations used: PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism; RE: Restrict enzyme; ITS: Internal transcribed spacer; RAPD: Random amplified polymorphic DNA analysis; AFLP: Amplified fragment length polymorphism.

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INTRODUCTION

The dried bark of *Cinnamomum cassia* Presl. (*Cc*), commonly known as "cinnamon," is an important medicinal crop that is widely used throughout the world as an ingredient in food and for medical applications. Cinnamon has a sweet taste and is used as a spicy ingredient in the food industry. *Cinnamomum osmophloeum* Kanehira. (*Co*) is a tree species that is indigenous to Taiwan and has been frequently planted in forests for landscaping or medicinal applications. Pharmacological studies have reported that *Co* has antifungal, anti-inflammatory, antitermitic, antibacterial, and antioxidative effects and reduced serum uric acid levels.[1-5] Research investigating the plant's phytochemical constituents has also shown that cinnamaldehyde makes 76% of essential oil in *Co*, which is higher than in other *Cinnamomum* species.[4] Therefore, *Co* essential oil may have high value added for the production of cosmetics or other related healthy products. Recently, various commercial products using *Co* as the functional ingredient have been created and marketed in Taiwan, including herbal tea, beverages, soap, and cosmetics.[6]

Cinnamomum burmannii (*Cb*) is also a member of the *Lauraceae* and has morphological features that are highly similar to those of *Co*. Therefore, *Cb* is commonly used as an adulterant of *Co* for planting.[6]

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In recent years, several studies have reported that various methods have been applied to the authentication of *Cinnamomum* species. The histological approach was concluded using microscopy, morphological identification, and phytochemical chromatographic analysis.[7-9] However, identification based on morphological characteristics has relied heavily on the researcher's experience or operation skills, dramatically reducing the efficiency of identification and its precision.[10] Moreover, environmental and other factors, such as growth, climate, and geography, may affect the morphological features and/or the chemical constituents of *Cinnamomum*; these effects can result in imprecise species identification using the above traditional analysis.[6] Currently, several DNA-based methods for plant identification have been developed, including random amplified polymorphic DNA analysis, amplified fragment length polymorphism, polymerase chain reaction (PCR), PCR restriction fragment length polymorphism (PCR-RFLP), authentication by sequencing of internal transcribed spacers (ITS) regions within the nuclear ribosomal DNA (rDNA), and authentication by sequencing of the chloroplast *trnL* DNA.[11-17] However, to date, few genetic identification studies focusing on *Cinnamomum* species have addressed the authentication of trees or the identification of their misused adulterants.[17]

For the reasons outlined above, the specific aim of this study was to develop a PCR-RFLP method for the rapid identification of *Co* from its adulterant *Cb* and related *Cinnamomum* plants by employing the ITS regions of the nuclear rDNA as a DNA molecular marker. In this study, a DNA restriction endonuclease (RE) pattern was successfully established, providing a more convenient and feasible way to authenticate the *Cinnamomum* species. To the best of our knowledge, this study is the first to describe the use of RE DNA patterns and the PCR-RFLP system for the genetic identification of *Co* and its popular adulterants.

MATERIALS AND METHODS

Plant source

Seven *Cinnamomum* species, *Co*, *Cb*, *Cinnamomum kanehirae (Ck)*, *Cinnamomum camphora (Ca)*, *Cinnamomum reticulatum (Cr)*, *Cinnamomum kotoense (Ckk)*, and *Cc*, were collected at different localities around Taiwan. The collection points of these specimens are presented in Table 1. The plant samples of these *Cinnamomum* species were identified by Dr. Wen-Te Chang, and voucher specimens were deposited at the Graduate Institute of Chinese Pharmaceutical Science and Chinese Medicine Resources of China Medical University. Voucher specimen numbers are denoted and listed in Table 1.

Table 1: Collected *Cinnamomum* species in this study

Scientific name	Collection localities	Abbreviation	Voucher number
Co	Taiching	CoD	CMR2011051
	Tainan	CoS	CMR2011052
	Kaohsiung	CoK	CMR2011053
	Pingtung	CoP	CMR2011054
	Nantou	CoL	CMR2011055
Cb	South Taichung	CbT	CMR2011061
	North Taichung	CbZ	CMR2011062
	Chunghwa	CbL	CMR2011063
Cinnamomum kanehirae	Miaoli	Ck	CMR2011056
Cinnamomum camphora	Nantou	Ca	CMR2011057
Cinnamomum reticulatum	Pingtung	Cr	CMR2011059
Cinnamomum kotoense	Taitung	Ckk	CMR2011058
Cinnamomum cassia	Chunghwa	Cc	CMR2011064

Cb: *Cinnamomum burmannii; Co: Cinnamomum osmophloeum*

DNA extraction

Dried leaves from the *Cinnamomum* samples were collected and ground with liquid nitrogen into powder to allow DNA extraction. Total DNA was isolated from each sample of homogenized plant tissue using a Geneaid Genomic Extraction Mini Kit (Geneaid, Taipei, Taiwan) according to the manufacturer's instructions. The concentration of purified total genomic DNA was determined using a spectrophotometer (NanoVue™, GE Healthcare, USA), and each sample was then stored at −20°C.

Primers

The specific oligonucleotide primers TCM-5 (5'-CGTAA CAAGGTTTCCGTAGGTGAAC-3') and TCM-12 (5'-GACG CTTCTCCAGACTACAA-3') were designed based on the 18S and 26S rDNA sequences of various *Cinnamomum* species, respectively.[6] The whole ITS DNA region, including ITS1, 5.8S RNA, and ITS2, was amplified when TCM-5 and TCM-12 were used as primers in the PCR amplification.[6]

DNA amplification and internal transcribed spacer sequence alignment

To align the ITS sequence, the complete ITS regions of the seven *Cinnamomum* species were amplified using the TCM-5 and TCM-12 primers with genomic DNA from these various *Cinnamomum* species as template DNA. In brief, 50 ng of total genomic DNA from each *Cinnamomum* species was individually added to the PCR mixture. PCR was performed in a 25-µl reaction mixture containing 0.4 mM of dNTPs, 5 pmol each of TCM-5 and TCM-12, 1U Pro-Taq™ DNA polymerase (Protech, Taiwan), and 1× Pro-Taq™ buffer (10 mM Tri-HCl, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 0.1% Triton X-100, pH 9.0). The PCR conditions were 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 57.7°C for 1 min, and 72°C for 1 min and a final extension cycle at 72°C for 10 min.[6] The amplified PCR products were resolved by 2% agarose gel electrophoresis. DNA banding was detected by the observation of the presence of visible DNA bands after staining with ethidium bromide. To sequence the ITS DNAs, each PCR product was individually added to the pGEM™-T vector, and TA cloning was carried out according to the manufacturer's instructions (Promega, USA) using T4 DNA ligase at 4°C with overnight incubation. The ligation mixture was transformed into competent *Escherichia coli* Top10 cells and recombinant colonies selected followed by plasmid purification and DNA sequencing. The sequences obtained for each ITS DNA region were then subjected to multiple pairwise sequence alignment using Clustal W2 software (http://www.ebi.ac.uk/clustalw/index.html).

Restriction analysis of the polymerase chain reaction products

The individual PCR products amplified by TCM-5 and TCM-12 were extracted from the agarose gel after DNA electrophoresis and purified using a Gel/PCR DNA fragment extraction kit according to the manufacturer's instructions (Geneaid, Taiwan). Digestion was carried out at 37°C for 1 h in a 50 µl RFLP reaction mixture containing 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, 100 ug/ml BSA, 5 U *Mly*I, and 5 U *Eco*RV. The resulting restriction digestion product was analyzed by 3% agarose electrophoresis and visualized using ethidium bromide staining and ultraviolet excitation.

RESULTS

Polymorphism of the internal transcribed spacer of the nuclear ribosomal DNA of various *Cinnamomum* species

To compare the interspecies sequence variation of *Cinnamomum* spp., the ITS regions of the different *Cinnamomum* spp. were used as targets for PCR amplification. Using the primers TCM-5 and TCM-12, a major single PCR product of approximately 800 bp was observed after DNA amplification from each *Cinnamomum* species [Figure 1a]. Sequencing of these ITS fragments, as illustrated in Figure 2, showed that these obtained ITS DNA regions of five *Co* from different localities with 790 bp in size demonstrated these sequences, including partial 18S and 26S rDNA, a complete 5.8S rDNA, ITS1, and ITS2. All ITS DNA of five *Co* sequences was identical. The other six *Cinnamomum* species, *Cb*, *Ck*, *Ca*, *Cr*, *Ckk*, and *Cc*, have ITS regions of 770 bp, 811 bp, 792 bp, 808 bp, 805 bp, and 800 bp, respectively. All obtained sequences of different *Cinnamomum* species were aligned to determine their similarities and to recognize their restriction maps using the software NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/). As illustrated in Figure 2, the results showed that a number of *Mly*I restriction sites were present in the abovementioned six *Cinnamomum* species, except in the sequence of *Cc*. Moreover, a unique *Eco*RV restriction site was only present in the sequence of *Ca* [Figure 2]. No *Eco*RV restriction sites were present in the other six *Cinnamomum* species. Thus, two restriction enzymes, *Mly*I and *Eco*RV, spanned in these ITS sequences may have the potential to be molecular markers for use in specific species identification of these seven *Cinnamomum* plants by PCR-RFLP analysis.

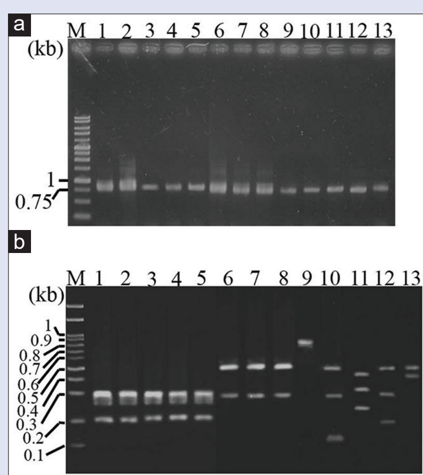

Figure 1: (a) Amplification of internal transcribed spacer DNA from various *Cinnamomum* plants by polymerase chain reaction. Polymerase chain reaction was performed using universal primers, TCM-5 and TCM-12, designed based on the sequence of nuclear ribosomal DNA. Polymerase chain reaction conditions were described in the methods and materials. (b) Polymerase chain reaction-restriction fragment length polymorphism analysis. *Myl*I and *Eco*RV restriction pattern of a polymerase chain reaction product of internal transcribed spacer DNA from seven Cinnamomum plants were resolved using 3% agarose gel. Lanes 1–5 indicate *Cinnamomum osmophloeum*; lanes 6–8 indicate *C. burmannii*; lanes 9–13 indicate *Cinnamomum kanehirae*, *Cinnamomum camphora*, *Cinnamomum reticulatum*, *Cinnamomum kotoense*, and *Cinnamomum cassia*, respectively. Lane M: DNA marker

Polymerase chain reaction-restriction fragment length polymorphism DNA polymorphism of the internal transcribed spacer regions from the seven *Cinnamomum* species

To rapidly identify these seven *Cinnamomum* plants using PCR-RFLP analysis, the restriction enzyme patterns of the seven species are predicted and listed in Table 2. The predicted restriction profiles suggested that if only one restriction enzyme, *Mly*I, is used, it appears not to be possible to clearly differentiate all the *Cinnamomum* species simultaneously in this study. Using *Mly*I, it was enabled to theoretically verify *Co* between *Cb*, *Ck*, *Ca*, *Cr*, *Ckk*, and *Cc* in terms of digestion pattern of restriction enzyme. However, the predicted restriction profiles with *Mly*I between *Ca* and *Ckk* have quite similar DNA banding patterns after electrophoresis. For the *Ca* sample, a 348-bp DNA fragment and a 460-bp DNA fragment were produced; similarly, a 346-bp DNA fragment and a 459-bp DNA fragment were produced in the *Ckk*. Based on this prediction, another restriction enzyme, *Eco*RV, was selected by the prediction software for use to improve the discrimination. As illustrated in Figure 1b, the restriction patterns created by *Mly*I and *Eco*RV showed that these DNA fragments were varied and clearly verified above seven *Cinnamomum* plants. The obtained RFLP patterns were notably different from those of the other *Cinnamomum* species, especially regarding the identification of *Co* and *Cb*. Taken together, RFLP analysis with *Mly*I and *Eco*RV established in this work is powerful and has the potential to be applied for molecular identification among popular adulterated *Cinnamomum* plants, especially for discriminating *Co* and *Cb*.

DISCUSSION

Generally, most *Cinnamomum* plants are highly economically valuable tree species. However, *Cinnamomum* species share similar morphological features in the taxonomy. Thus, developing a rapid and feasible method for the identification of *Cinnamomum* plants is needed to prevent their adulteration of trees. In Taiwan, due to the applications of this plant to food, cosmetic, and medical usage, *Co* is widely planted by farmers in the field. However, in Taiwan, tree adulteration of *Cb* during planting results in significant economic losses in agriculture.

In this study, we have successfully developed a PCR-RFLP method for the rapid and specific discrimination of *Co* trees from other *Cinnamomum* species samples. Previous studies have reported that many genetic markers, such as ITS of the rDNA region and specific genes in the chloroplast DNA, could be used for molecular identification.[17,18] Among these markers, ITS has strong potential for use as a barcode for plant authentication.[18] However, which DNA regions or specific genes are best or suitable for molecular identification depends on the species in the family. In this study, DNA from the ITS region was chosen for

Table 2: Predicted restriction fragments in sizes (in bp) among *Cinnamomum* species when restriction enzymes were used for digestion

Scientific name	Digestion with restriction enzyme (produced fragments in different sizes)	
	***Mly*I**[a]	***Mly*I + *Eco*RV**[b]
Co	280, 300, 210	280, 300, 210
Cb	500, 270	500, 270
Cinnamomum kanehirae	114, 234, 463	114, 234, 463
Cinnamomum camphora	348, 460	78, 270, 460
Cinnamomum reticulatum	194, 244, 334	194, 244, 334
Cinnamomum kotoense	346, 459	346, 459
Cinnamomum cassia	800	800

[a]Predicted restriction fragments of different sizes, [b]The experimental restriction fragments produced after digestion with *MlyI* and *EcoRV* simultaneously

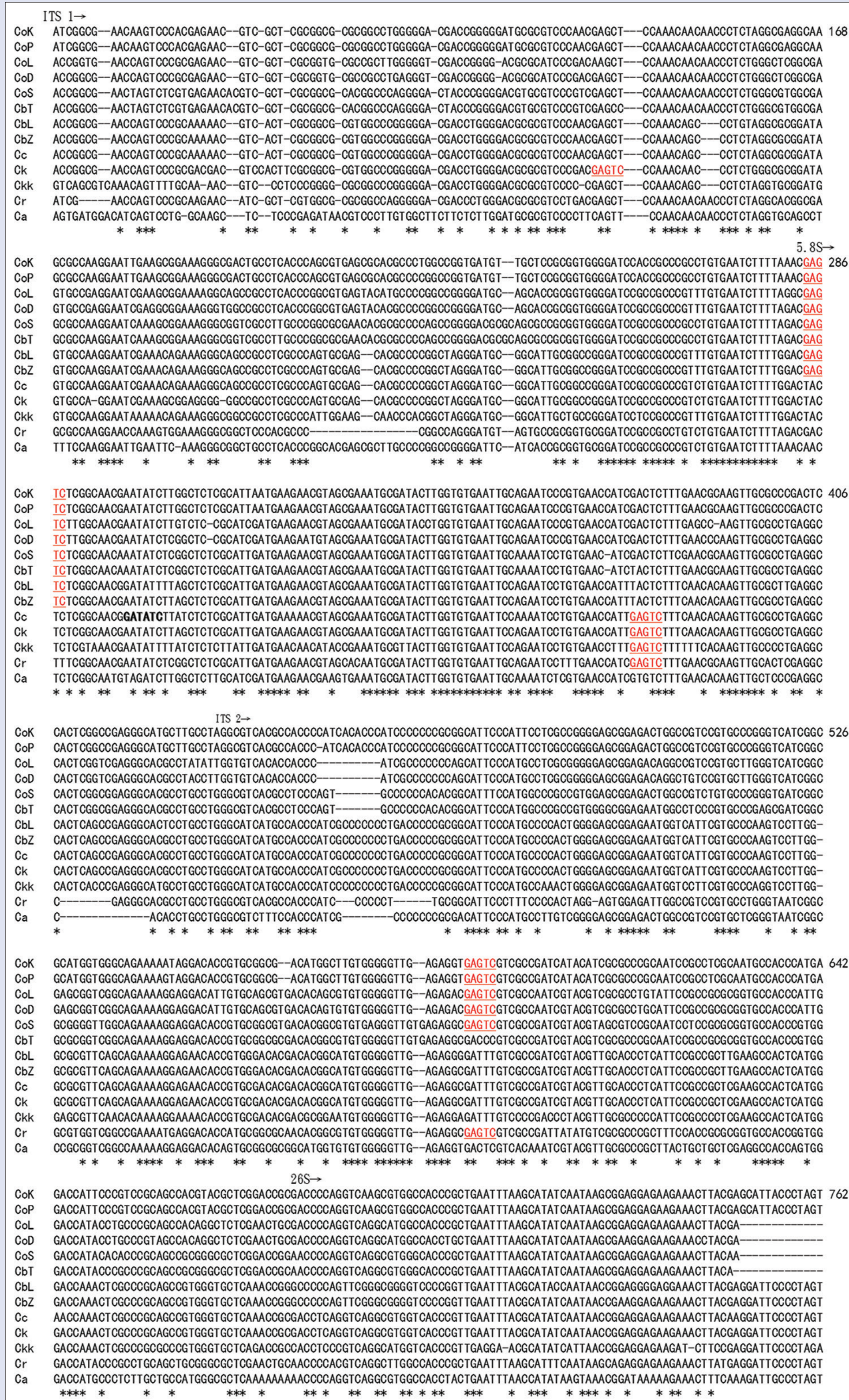

Figure 2: Sequence comparison of a region of internal transcribed spacer of nuclear ribosomal DNA for seven *Cinnamomum* plants. An asterisk (*) represents the aligned nucleotide that is identical to the upper sequence. A hyphen (-) represents a gap in the aligned sequence. The restriction enzyme sites of *Myl*I and *Eco*RV are indicated by underline and bold, respectively

amplification using PCR primers. Sequence alignment analysis of the ITS regions from seven *Cinnamomum* species revealed that the ITS1 and ITS2 regions showed higher interspecies variation than intraspecies. In contrast, the sequence divergence across the *Cinnamomum* species was not great. Therefore, based on the sequence variation of ITS, it is not easy to design species-specific PCR primers for use in direct identity

identification via a diagnostic PCR method. Normally, sequencing of an ITS DNA fragment during medicinal herb identification is a useful approach, and it directly overcomes the problem of species identification. However, a lack of truly universal primers for the amplification of ITS regions from a wide variety of plants has limited the usefulness of this approach as a general method of species identification.[18] Moreover, the direct sequencing of a PCR product is a relatively costly procedure and is time-consuming. To improve such problems, other molecular techniques, such as PCR-RFLP, have been developed and used for species identification; such an approach requires a good universal primer pair for the amplification of the ITS region of *Cinnamomum* species for further organization of genetic information.[10,19,20] In most plants, the length of the ITS1-5.8S rDNA-ITS2 region ranges from 550 to 850 bp.[18,21,22] The present studies showed that the length of the ITS regions of the various *Cinnamomum* species varied [Figure 2]. Nevertheless, the *Cinnamomum* universal primers, TCM-5 and TCM-12, were able to conveniently amplify the ITS DNA regions of the seven *Cinnamomum* species and thus seem to be both compatible with and effective for ITS amplification from *Cinnamomum* samples in general.

As to the analysis on the investigation of the restriction maps of the ITS regions of seven *Cinnamomum* plants, *Mly*I was selected as a candidate restriction enzyme that might be useful when discriminating between *Co* and other *Cinnamomum* species [Table 2]. However, the predicted restriction profiles of *Ca* and *Ckk* with *Mly*I were highly similar and difficult to distinguish their differences from each other in terms of conventional DNA banding by agarose gel electrophoresis (data not shown). As a result, digestion of the PCR product of ITS DNA with *Mly*I and *Eco*RV was performed for the experiment. As expected, this enzyme combination is capable of distinguishing between *Ca* and *Ckk*. It is worth noting that in this study, plant leaves were used to extract genomic DNA for tree identification in the farm field. In fact, the barks of *Cinnamomum* are being used in traditional medicine or in the food industry after agricultural harvesting. However, different regions of extracted DNA used in this study did not influence the molecular identification theoretically. Thus, this developed PCR-RFLP may not only be employed on tree authentication but may also be useful as an alternative way to identify cinnamon products.

CONCLUSION

The PCR-RFLP method described in the present study enabled us to rapidly and conveniently identify Co from various *Cinnamomum* species. It may be useful and easy to standardize this method, which can be applied practically for *Cinnamomum* identification. Moreover, in the future, it might be possible to apply this approach for the investigation of the population diversity and structure of *C. osmopholeum* and other *Cinnamomum* species that are indigenous to Taiwan.

Acknowledgements

Bo-Cheng Yang and Meng-Shiunn Lee contributed equally to this work.

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Conflicts of interest

There are no conflicts of interest.

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