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Noncoding Pastid tRNA-Leu (trnL) Intron Region Sequences Report for Genetic Separation of *Cinnamomum spp.* from China and Myanmar

Khin Thantsin

Abstract

To study the relationships in the genus *Cinnamomum*, 493 base pairs of the trnL intron region plastid gene have been sequenced and analyzed for 8 species, which collected from China and Myanmar. These 8 taxa are clearly grouped into two according to its location Myanmar and China. Furthermore, PCR product of the tRNA-Leu (trnL) intron results of *C. tamala, C. multiflorum, C. obtusifolium, C. inunctum* or related species were reported.

Introduction

Cinnamon is one of the oldest known spices and remains an important mercantile commodity today. Its appealing characteristic aroma and flavor finds many uses in the food, cosmetics and pharmaceutical industries (Farrell, 1999; Tainter, et al., 2001). The cinnamons of commerce are derived from the dried inner bark of several species of the genus *Cinnamomum* (Tainter, et al., 2001).

Genus *Cinnamomum* belongs to the Lauraceae and is composed of approximately 110 species of evergreen trees and shrubs (Purseglove, 1969) and can be distinguished from other members of Lauraceae by no petiolar sheath, periderm retaining a green shade on numerous successive internodes. All stem with spiral phyllotaxy (stem angular or grooved; some leaves opposite), young leaves reddish, venation not palmate (the two main lateral veins are inserted above the very based of the main vein) thus leaf is supratrinerved (Killer, 2004). However, this character combination is not found in all species.

In recent times contemporary systematic has put a greater emphasis on molecular rather than morphological data, the time seems ripe to reevaluate plant morphology and what its role can and should be in modern plant biology (Donald, 2001). There have been several reports of cpDNA analysis of Lauraceae species. However, there is no report for *C. tamala, C. multiflorum, C. obtusifolium* and *C. inunctum.*

Lecturer, Department of Botany, Myeik University

An important consequence of the sedentary lifestyle of plants is that they cannot escape from the environment in which they grow or from any changes in this environment. To cope with this, many plants are able to alter one or more morphological characters in response to both abiotic (e.g., climate and weather) and biotic (e.g., grazing and competition) factors of the environment with a potential effect on resource acquisition.

The samples of *Cinnamomum* taxa studied here have rather different geographical distributions. In this paper, we investigated the genetic separation of genus *Cinnamomum* between China and Myanmar taxa using sequences of tRNA-Leu (trnL) intron plastid gene and reported the tRNA-Leu (trnL) intron sequences for *C. tamala, C. multiflorum, C.obtusifolium, C. inunctum* and related species.

Material and method

Material

Samples of the species were collected from documented collections of botanic gardens. All these samples were identified systematically by Professor Dr. Wang Qiang and deposited in the Department of Chinese Materia Medica Analysis, China Pharmaceutical University, Nanjing, China. Eight samples of genus *Cinnamomum*, four from southern part of Yangzi River, China (*Cinnamomum burmannii, Cinnamomum heyneanum, Cinnamomum japonicum* and *Cinnamomum wilsonii*) and four from Ayeyawaddy delta, Myanmar (*Cinnamomum tamala, Cinnamomum multiflorum, Cinnamomum inunctum* and *Cinnamomum obtusifolium*) were used. Leaf tissue of all samples was collected in the field, dried with silica gel and stored at –20°C. The origins of the materials are shown in Table 1.

10×PCR reaction buffer, 25 mM $MgCl_2$, *Taq* DNA polymerase (Promega), dNTPs Mix and DNA Purification Kit (Sangon), primers synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.; PTC-200 thermocycler (MJ Research), ABI Prism 310 Genetic Analyzer.

Table 1 List of the investigated *Cinnamomum spp.* with their geographical distribution and the genbank accession numbers

No.	Name of species	Boucher no. and locality	Accession no. in Genbank
1	*C. tamala* Fr.Nees	KT2~1,Yangon,Myanmar	DQ822585
2	*C. multiflorum* Wight	QDQ~16,Yangon,Myanmar	DQ822586
3	*C. inunctum* Meissen	QDQ~17,Yangon,Myanmar	DQ822587
4	*C. obtusifolium* (Roxb.) Nees	QDQ~20,Yangon,Myanmar	DQ822588
5	*C. japonicum* Sieb.	QDQ~3, Nanjing, China	DQ822589
6	*C. heyneanum* Nees.	QDQ~12, Guangzhou, China	DQ822590
7	*C. burmannii*(C. G. & Th. Nees) Bl	QDQ~13,Guanzhou, China	DQ822591
8	*C. wilsonnii* Gamble.	QDQ~5, Nanjing, China	DQ822592

Method

Total genomic DNA was extracted from fresh leaf by a protocol modified from Rogers'method. Amplification of cpDNA was carried out in 30 μl reaction volumes containing 3 μl 10×PCR reaction buffer, 1.5 μl 25 mM $MgCl_2$, 2 μl 2 mM dNTPs Mix, 1.0 unit of *Taq* DNA polymerase, 1 μl 10 μM forward and reverse primers and approximately 70-80 ng of total DNA. The forward primer (5′-GGTTCAAGTCCCTCTATCCC-3′) and reverse primer (5′-ATTTGAACTGGTGACACGAG-3′) were used for amplification of cpDNA (Kojoma et al., 2002). The profile for the cycles of amplification was: an initial 4 min at 95°C followed by 30 sec at 95°C for denaturation, 45 sec at 54°~57° C for primer annealing and 1 min at 72°C for primer extension, repeated for 30 cycles, and a final extension of 10 min at 72° C. PCR reaction was carried out by a PTC-200 thermocycler. PCR products were purified by means of DNA Purification Kit. DNA products and the purified products were detected by ethidium bromide staining under UV after electrophoresis in 1.0 % agarose gel. According to the manufacturer's instruction, the purified PCR products were sequenced with the BigDye terminator mix on an ABI Prism 310 Genetic Analyzer. A forward primer was used to sequence all samples. The complete alignment was done with Clustal X 1.8 (Aiyar, A., 2000) and refined manually.

Result

The nucleotide sequence data reported in this paper will appear in the Genbank nucleotide sequence databases, with the accession number shown in the Table 1. Variable sites of trnL intron region sequences from 8 species of Genus *Cinnamomum* are as follow.

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C. tamala        -------GGA ACCTACTAAG TGATAACTC
C. multiflorum   TTGGTAT... .......... .........
C. inunctum      -TGGTAT... .......... .........
C. obtusifolium  ------T... .......... .........
C. japonicum     ---------- ---------- -T.AC.T..
C. heynaenum     ---------- ---------. AT.AC.T..
C. burmannii     ---------- ---------- --------.
C. wilsonii      ---------- ---------- -T.AC-T..

C. tamala        CAAATTCAGA GAAACCCTGG AATTAAAAAT
C. multiflorum   .......... .......... ..........
C. inunctum      .......... .......... ..........
C. obtusifolium  .......... .......... ..........
C. japonicum     .......... .......... ..........
C. heynaenum     .......... .......... ..........
C. burmannii     .......... .......... ..........
C. wilsonii      .......... .......... ..........

C. tamala        GGGCAATCCT GAGCCAAATC CTGTTTTCAG
C. multiflorum   .......... .......... ..........
C. inunctum      .......... .......... ..........
C. obtusifolium  .......... .......... ..........
C. japonicum     .......... .......... ..........
C. heynaenum     .......... .......... ..........
C. burmannii     .......... .......... ..........
C. wilsonii      .......... .......... ..........

C. tamala        AAAACAAGGG TTCAGAAAGC GAGAACCAAA
C. multiflorum   .......... .......... ..........
C. inunctum      .......... .......... ..........
C. obtusifolium  .......... .......... ..........
C. japonicum     .......... .......... ..........
C. heynaenum     .......... .......... ..........
C. burmannii     .......... .......... ..........
C. wilsonii      .......... .......... ..........
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C. tamala         AAAGGATAG GTGCAGAGAC TCAAAGGAAG
C. multiflorum    ...|..... ......... .........
C. inunctum       ...|..... ......... .........
C. obtusifolium   ...|..... ......... .........
C. japonicum      ...|..... ......... .........
C. heynaenum      ...|..... ......... .........
C. burmannii      ...|..... ......... .........
C. wilsonii       ...|..... ......... .........

C. tamala         CTGTTCTAAC GAATGGAGTT GATTAACATT
C. multiflorum    .......... .......... ..........
C. inunctum       .......... .......... ..........
C. obtusifolium   .......... .......... ..........
C. japonicum      .......... .......... ..........
C. heynaenum      .......... .......... ..........
C. burmannii      .......... .......... ..........
C. wilsonii       .......... .......... ..........

C. tamala         GGTATAGGAA TCCTTCTATC GAAATTCCAG
C. multiflorum    .......... .......... ..........
C. inunctum       .......... .......... ..........
C. obtusifolium   .......... .......... ..........
C. japonicum      .......... .......... ..........
C. heynaenum      .......... .......... ..........
C. burmannii      .......... .......... ..........
C. wilsonii       .......... .......... ..........

C. tamala         AAAGGATGAC CCTATCCTAT ATACGTACTG
C. multiflorum    .......... .......... ..........
C. inunctum       .......... .......... ..........
C. obtusifolium   .......... .......... ..........
C. japonicum      .......... .......... ..........
C. heynaenum      .......... .......... ..........
C. burmannii      .......... .......... ..........
C. wilsonii       .......... .......... ..........

C. tamala         AAATATCAAA CAATTAATCA CGATCCGATT
C. multiflorum    .......... .......... ..........
C. inunctum       .......... .......... ..........
C. obtusifolium   .......... .......... ..........
C. japonicum      .......... .......... ..........
C. heynaenum      .......... .......... ..........
C. burmannii      .......... .......... ..........
C. wilsonii       .......... .......... ..........
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```
C. tamala        CCTATTTTT TTTATATGAA AAATGGAAGA
C. multiflorum   ......    .......... ..........
C. inunctum      ......    .......... ..........
C. obtusifolium  ......    .......... ..........
C. japonicum     ......    .......... ..........
C. heynaenum     ......    .......... ..........
C. burmannii     ......    .......... ..........
C. wilsonii      ......    .......... ..........

C. tamala        ATTCTTGGA ATCGATTCCA AATTGAAGGA
C. multiflorum   .......   .......... ..........
C. inunctum      .......   .......... ..........
C. obtusifolium  .......   .......... ..........
C. japonicum     .......   .......... ..........
C. heynaenum     .......   .......... ..........
C. burmannii     .......   .......... ..........
C. wilsonii      .......   .......... ..........

C. tamala        AGAATCGAAT ATTCAGTGAT CAAATCATTC
C. multiflorum   .......... .......... ..........
C. inunctum      .......... .......... ..........
C. obtusifolium  .......... .......... ..........
C. japonicum     .......... .......... ..........
C. heynaenum     .......... .......... ..........
C. burmannii     .......... .......... ..........
C. wilsonii      .......... .......... ..........

C. tamala        ACTCCTCGGA TAGATCTTTT GAAGAACTGA
C. multiflorum   .......... .......... ..........
C. inunctum      .......... .......... ..........
C. obtusifolium  .......... .......... ..........
C. japonicum     .......... .......... ..........
C. heynaenum     .......... .......... ..........
C. burmannii     .......... .......... ..........
C. wilsonii      .......... .......... ..........

C. tamala        TTAATCGGAC GAGAATAAAG ATAGAGTCCA
C. multiflorum   .......... .......... ..........
C. inunctum      .......... .......... ..........
C. obtusifolium  .......... .......... ..........
C. japonicum     .......... .......... ..........
C. heynaenum     .......... .......... ..........
C. burmannii     .......... .......... ..........
C. wilsonii      .......... .......... ..........
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```
C. tamala        TTCTACATGT CAATACCGAC AACAATGAAA
C. multiflorum   .......... .......... ..........
C. inunctum      .......... .......... ..........
C. obtusifolium  .......... .......... ..........
C. japonicum     .......... .......... ..........
C. heynaenum     .......... .......... ..........
C. burmannii     .......... .......... ..........
C. wilsonii      .......... .......... ..........

C. tamala        TTTATAGTAA GGGGAAAATC CGTCGACTTT
C. multiflorum   .......... .......... ..........
C. inunctum      .......... .......... ..........
C. obtusifolium  .......... .......... ..........
C. japonicum     .......... .......... ..........
C. heynaenum     .......... .......... ..........
C. burmannii     .......... .......... ..........
C. wilsonii      .......... .......... ..........

C. tamala        AGAAATCGTG AGG
C. multiflorum   .......... ...
C. inunctum      .......... ...
C. obtusifolium  .......... ...
C. japonicum     .......... ...
C. heynaenum     .......... ...
C. burmannii     .......... ...
C. wilsonii      .......... ...
```

Figure 1 Variable sites of trnL intron region sequences from 8 species of Genus *Cinnamomum* (Taxa names are shown on the left, a dot indicates identity sites)

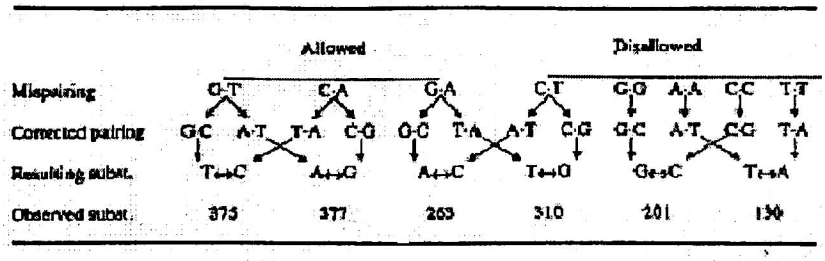
C. tamala, C. multiflorum, C. obtusifolium and *C. inunctum* which collected from Yangon, Myanmar and *C. burmannii, C. heyneanum* collected from Guanzhou, *C. japonicum, C. wilsonii* collected from Nanjing, China. They showed obviously 4 nucleodide substitutions among them.

Discussion

According to the result, location 29, 273 and 298 indicated gene substitution between China taxa and Myanmar taxa. Within the 4 Myanmar taxa, *C. inunctum* is a section 1. camphora member; the other 3 are members of section 2. cinnamomum. Even though section 1 and section. 2 are taxonomically far from each other, but in our study their genetic data did not show any sequence divergence in trnL intron region. Visible divergence was found only in different location between China and Myanmar taxa (Fig. 1). The data address us abiotic and biotic factors of the environment with a potential effect on resource acquisition of genetic differences. Authentic variable sites have been found in the trnL intron region sequences of *Cinnamomum spp.* for China and Myanmar taxa. The sequence of the four species from Myanmar was a "G" at position 273 and 298, while in four China taxa there was a "T". The point mutation (position 273, 298) was recognized as the molecular marker for distinguishing the *Cinnamomum spp.* of China and Myanmar.

Angiosperm chloroplast DNA studies have focused mainly on substitution patterns in genes (Albert et al., 1994; Catalan et al., 1997; Hilu and Liang, 1997; Kellogg and Juliano, 1997; Manen et al., 1998). In spite of the high A+T content in the trnL-trnF regions, transversion were found to occur significantly less often than the other substitution types in the intron + spacer regions. A+T content of the angiosperm alignments of the total trnL-F region ranged between 64.5% and 67.1% (Bakker et al., 2000). In the present study, A + T content was 63.9% and 4 nucleotide substitutions were observed, within these 4 substitutions, transversion was found only in location 29. A + T content 63.9% also indicates high % of A+T content.

Base mispairings were similar (allowed) or non-similar (disallowed) in geometry to standard Watson-Crick base pairs. The following figure showed over view of routes leading from base mispairing to substitution in DNA sequences during replication (Echols and Goodman, 1991).



Recent study observed only one location (location124) showed similar (allowed) base pairing to substitution and other 3 location (location 29,273 and 298) indicated non-similar (disallowed). Within these 3 non-similar (disallowed), only at location 29 has transversion.

According to the Kojoma, et al. 2002, 6 trnL intron region sequences of *C. cassia* which collected from Japan, China and Vietnam were reported as identical. In our present study, the trnL intron region sequences showed base pair substitution in different location. Furthermore, 12 sequences, 4 from Japan (Kojoma, et al. 2002) and 8 from present study were done complete alignment with Clustal X 1.8 and the nucleotide substitution between Japan, China and Myanmar was obvious.

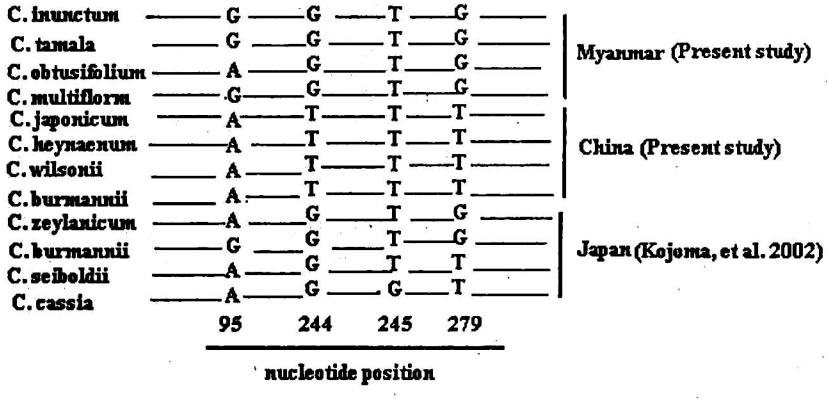


Figure 2 Summarized sequence variation of noncoading plastid tRNA-Leu (trnL) intron region sequences of *Cinnamomum* (Taxa names are shown on the left)

The above figure was summarized sequence variation of noncoading plastid tRNA-Leu (trnL) intron region sequences of *Cinnamomum* species which collected from 3 different countries. This figure gave a short message that taxa from different location even the same species (*C. burminnii* in here) have a nucleotide substitution in trnL intron region.

Even though these 8 species possessed different nucleotide sequences, information from the molecular analyses of an organelle alone is not sufficient to prove hybridization. But when taxa are suspected of involvement with hybrids, the combined data from these different approaches can provide powerful insights (Isoda et. al., 2000). Even though there are many literatures indicated the trnL-trnF and the trnL intron can be used to distinguish in species level, our data address the abiotic and biotic factors of the environment with a potential effect on resource acquisition of genetic differences. It is hoped that further studies will benefit from the results presented here and that a comprehensive analysis of the entire genus.

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