

Identification of *Murraya koenigii* (L.) Spreng Using DNA Barcoding Technique Based on the ITS Sequence

Worapan Sitthithaworn*, Lalita Wirasathien, Lalida Kampiranont, Panisara Khomdej and Walalee Waiyasilp

Faculty of Pharmacy, Srinakharinwirot University, Nakonnayok, 26120 Thailand

* Corresponding author: worapan@swu.ac.th

ABSTRACT

OBJECTIVE: To determine pharmacognostical characteristics and DNA marker of *Murraya koenigii* leaf. **METHODS:** Leaf tissue of *M. koenigii* was investigated under light microscope. DNA marker for *M. koenigii* was established by amplification of the internal transcribed spacer (ITS) of nuclear ribosomal RNA gene using conserved plant sequences as primers. Multiple alignment with ITS sequences of plants in the tribe Clauseneae retrieved from GenBank was then performed to identify the polymorphic sites. **RESULTS:** Microscopic results showed the presence of calcium oxalate prism sheath, unicellular trichomes, anisocytic type of stomata and the schizolysigenous oil glands. An alignment result of ITS sequences indicated several polymorphic sites that could be exploited as DNA markers and revealed the presence of a *Bsr*BI restriction site in *M. koenigii* which could be used to cut the PCR amplified DNA segment to generate fingerprint for identification of *M. koenigii*. **CONCLUSION:** Microscopic investigation of *M. koenigii* leaf exhibited characteristics of *Murraya* species and the ITS sequence was an appropriate molecular marker for discrimination of *M. koenigii* from the selected plants.

Keywords: *Murraya koenigii*, microscopic investigation, DNA marker, internal transcribed spacer

Thai Pharm Health Sci J 2010;5(3):202-205[§]

Introduction

Murraya koenigii (L.) Spreng commonly known as curry leaf, a plant in family Rutaceae, is used as a culinary herb and traditional medicine in India as antiemetic, anti-diarrhoeal and febrifuge. The oil derived from the leaves is also traditionally applied externally to bruises and eruptions.¹ The oil is also used in the perfume and soap industries. In Thailand, the plant is known as Hom Kaek and its leaf is used mainly as a flavoring agent in Indian curries. Pharmacological studies indicated that it possesses antioxidant, antibacterial, anticarcinogenic, anti-lipid peroxidative, hypoglycemic and hypolipidemic properties. Recently, five alkaloids namely euchrestine B, bismurrayafoline E, mahanine, mahanimbicine, and mahanimbine of carbazole type were extracted from the leaves and these alkaloids were reported to possess antioxidant activity.² Gas chromatography mass spectroscopy results revealed that

volatile oil extracted from *M. koenigii* leaves from Bangladesh contained 3-carene as a major compound.³

Based on the positive pharmacological activities, *M. koenigii* is gaining potential for herbal therapeutic products. Although the leaf of *M. koenigii* is characterized by being compound leaf with asymmetrical base and strong odiferous oil, the commercial raw material which can be sold in the form of shredded slice or dried powder makes it difficult to be differentiated by the appearance. Contamination with similar looking plants and adulteration of some commercial samples also cause problems for identification. Due to these problems, the establishment of standards for authentication of *M. koenigii* is important to ensure the efficacy. Molecular techniques are now being utilized increasingly to authenticate medicinal plants because DNA profiles have several advantages over morphological and chemical analyses. This is because genotype is unique for a given species and not affected by age, physiological conditions or environmental factors.⁴ A number of studies indicated the

[§] 15th year of Srinakharinwirot Journal of Pharmaceutical Science

excellent applications of the internal transcribed spacer (ITS) of nuclear ribosomal RNA gene (rDNA) for authentication of several species of plants⁵⁻⁸ as it evolves relatively quickly in producing sequence variation between species. ITS is non coding region separating three ribosomal subunits (Figure 1). ITS1 locates between 18S and 5.8S rDNA and ITS2 between 5.8S and 26S rDNA.

In the present study, we examined some pharmacognostic characteristics of the leaf of *M. koenigii* as well as DNA fingerprint patterns by means of sequencing of ITS DNA with subsequent comparison of the nucleotide sequence data to other selected plants in the Rutaceae family. The results of the study could be useful in setting some diagnostic indices for the identification and preparation of a monograph of the plant.

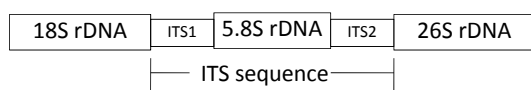


Figure 1 Structure of nuclear ribosomal RNA gene (rDNA).

Materials and Methods

Plan materials

Leaves of *Murraya koenigii* (L.) Spreng were collected from the botanical garden of Faculty of Pharmacy, Srinakharinwirot University, Nakonnayok, Thailand. Voucher specimens were deposited in the herbarium of the same institute.

Microscopic analysis

The leaf of *M. koenigii* was boiled with saturated chloral hydrate solution for microscopical observation.⁹

Amplification of ITS sequence using polymerase chain reaction and establishment of DNA marker

DNA was extracted from 100 mg of fresh leaves of *M. koenigii* using a DNeasy Plant Mini kit (Qiagen, Germany). The resulting DNA was then used as a template for PCR. Primers 18d (5'-CAC ACC GCC CGT CGC TCC TAC CGA-3') and 28cc (5'-ACT CGC CGT TAC TAG GGG AA-3') complementary to the conserved regions of plant 18S and

26S subunits of rDNA⁵ were used to amplify the region of ITS1-5.8S-ITS2 of rDNA (Figure 1). The PCR mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 1.5 U Taq polymerase (Invitrogen, USA) and 0.25 mM of each primer. The PCR program was hot started at 94 °C for 5 min, followed by 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min, for 30 cycles and a final extension at 72 °C for 10 min. After purification with a QIA quick PCR Purification Kit (Qiagen, Germany), DNA sequencing was performed with a Big Dye Terminator Kit Version 3.1 (Applied Biosystems) using a model ABI Prism 3100 Avant Genetic Analyzer (Hitachi). DNA sequences were analyzed using BioEdit Sequence Alignment Editor (www.mbio.ncsu.edu/BioEdit/bioedit.html). The ITS sequence of *M. koenigii* was then aligned using Clustal W 1.83 computer program (www.ebi.ac.uk/clustalw/) with ITS sequences of plants in the tribe Clauseneae retrieved from GenBank. DNA marker for *M. koenigii* was set up by identification of the polymorphic sites that presented in the ITS sequence of *M. koenigii*.

Results and Discussion

The microscopic studies of the leaf of *Murraya koenigii* showed the presentation of calcium oxalate prism sheath and thin-walled, long unicellular trichomes. The stomata were identified as anisocytic type and the schizolysigenous oil glands were observed prominently (Figure 2).

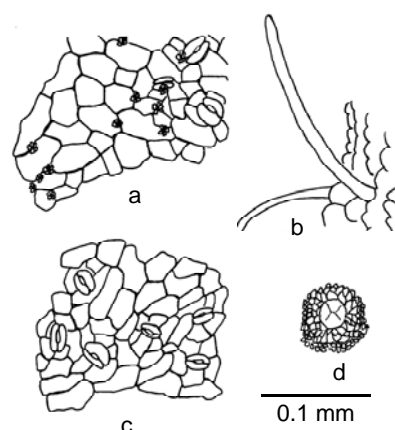


Figure 2 Microscopic characteristics of *Murraya koenigii* leaf: (a) calcium oxalate prism sheath; (b) unicellular trichome; (c) anisocytic type of stomata; and (d) schizolysigenous oil gland.

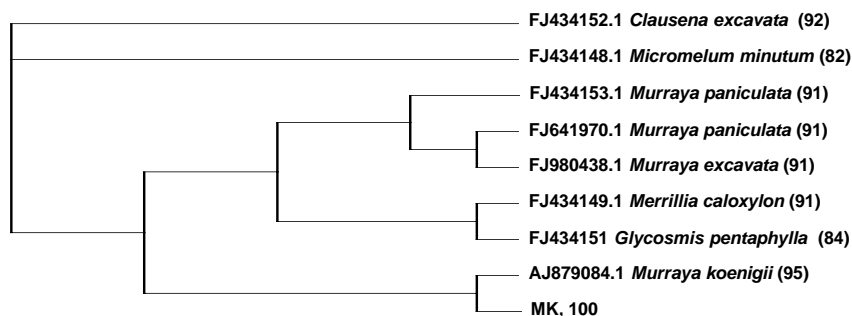


Figure 3 Phylogenetic tree analysis of ITS sequence from Clustral W 1.83 alignments, viewed using TreeView v 1.6.6. Accession numbers are indicated in front of the plant names. Numbers in brackets indicate the % identity from the PSI-BLAST.



Figure 4 The DNA sequences of *M. koenigii* in the ITS1-5.8S-ITS2 region 5.8S are boxed (MK denotes sequences from this study while MKG were those retrieved from GenBank). Arrows indicate restriction site presented only in *M. koenigii*. An asterisk (*) indicates consensus base.

In order to establish the DNA marker for *M. koenigii*, ITS sequence of *M. koenigii* obtained from this study designated as MK was compared to ITS sequences obtained from the plants in the tribe Clauseneae that were reported in Genbank. The tribe Clauseneae contains five genus namely *Murraya*, *Clausena*, *Glycosmis*, *Micromelum* and *Merillia*¹⁰ and until now there are eight ITS sequences that were reported in GenBank.¹¹ As shown in figure 3, the ITS

sequence from MK has 95% homology with ITS sequence of *M. koenigii* retrieved from GenBank (designated as MKG). Several polymorphic sites were observed in ITS1 region but a few polymorphic sites were found in 5.8s and ITS2 regions (Figure 4). The sequence variations among ITS DNA obtained from MK and the one obtained from MKG may be due to geographic variation. DNA polymorphism due to

geographic variation was also reported in *Curcuma zedoaria* from China and Japan.¹²

The polymorphic sites were greater when ITS sequences from MK and MKG were compared to the plants in the tribe Clauseneae. These polymorphic sites could be exploited as DNA markers for identification of *M. koenigii*. Restriction analysis suggested by a computer program (Webcutter 2.0, <http://rna.lundberg.gu.se/cgi-bin/cutter2/cutter>) revealed that a *Bsr*BI restriction site was present in the ITS2 region of MK and MKG but not in ITS sequences from the other selected plants (Figure 4). This suggested a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach for identification of *M. koenigii*. In PCR-RFLP, an appropriate restriction enzyme can be used to cut the PCR amplified DNA segment which generates species-specific fingerprint. Thus, *M. koenigii* can be distinguished from the selected plants by the presence of 2 fragments of DNA after PCR amplification of the ITS sequence followed by digestion with restriction enzyme *Bsr*BI.

Conclusion

Microscopic investigation of *M. koenigii* leaves revealed the presence of calcium oxalate prism sheath, unicellular trichomes, anisocytic type of stomata and schizolysigenous oil glands, which have been regarded as the characteristic of *Murraya* species.¹³ DNA studies indicated that DNA profile of *M. koenigii* are closely related to other plants with taxonomic relation, namely, *M. paniculata*, *M. excavata*, *Micromelum minutum*, *Merillia caloxylon*, *Glycosmis pentaphylla* and *Clausena excavate*. An alignment of ITS sequence indicated that several polymorphic sites were present throughout the sequence, particularly, in the ITS1 and ITS2 regions. Thus, *M. koenigii* is distinguishable from the selected plants by direct sequencing of the ITS DNA. It is also possible to discriminate *M. koenigii* from the selected plants by PCR-RFLP of the ITS DNA based on the presence of a *Bsr*BI restriction site.

Acknowledgement

This work was supported by Faculty of Pharmacy, Srinakharinwirot University (grant No. 208/2552).

References

1. Parmar C, Kaushal MK. *Murraya koenigii* (Accessed on Jan. 20, 2009, at <http://www.hort.purdue.edu/newcrop/parmar/12.html>)
2. Tachibana Y, Kikuzaki H, Lajis NH, Nakatani N. Antioxidative activity of Carbazoles from *Murraya koenigii* Leaves. *J. Agric. Food Chem* 2001;49(11):5589–5594.
3. Chowdhury JU, Bhuiyan MNI, Yusuf M. Chemical composition of the leaf essential oils of *Murraya koenigii* (L.) Spreng and *Murraya paniculata* (L.). *Bangladesh J Pharm* 2008;3(2):59-63.
4. Shaw P, Wang J, But P. Authentication of Chinese medicinal materials by DNA technology. Hong Kong. World Scientific, 2002: pp.1-23.
5. Ngan F, Shaw P, But P, et al. Molecular authentication of *Panax* species. *Phytochem* 1999;50:787-791.
6. Jobs D, Thien L. A conserved motif in 5.8S Ribosomal RNA (rRNA) gene is useful diagnostic marker for plant internal transcribed spacer (ITS) sequences. *Plant Mol Biol Reporter* 1997; 15:326-334.
7. Lau DT, Shaw PC, Wang J, et al. Authentication of medicinal *Dendrobium* species by the internal transcribed spacer of ribosomal DNA. *Planta Med* 2001;67:456-460.
8. Sitthithaworn W, Vimolmangkang S, Chittasupho C, Petcheunsakul D, Apa-a-dul S. Pharmacognostic investigation of the leaves of *Mentha cordifolia* and its DNA fingerprints. *Thai Pharm Health Sci J* 2009;4(1):9-14.
9. Evans WC. Trease and Evans' pharmacognosy, 14th ed. London. WB Saunders Company Ltd., 1996: pp.539-568.
10. Sohrab MH, Chowdhury R, Hasan MC, Rashid MA. Chemotaxonomic significance of polyoxygenated flavonoids from the leaves of *Micromelum minutum*. *Biochem Sys Ecol* 2004;32:829-831.
11. EMBL/GenBank/DDBJ databank accession numbers: FJ434152.1; FJ434153.1; FJ434148.1; FJ434149.1; FJ434151; FJ646970.1; FJ980438.1; AJ879084.1.
12. Sasaki Y, Fushimi H, Cao H, Cai SQ, Komatsu K. Sequence analysis of Chinese and Japanese curcuma drugs on the 18S rRNA gene and trnK gene and the application of amplification-refractory mutation system analysis for their authentication. *Biol Pharm Bull* 2002;25:1593-1599.
13. Kong YC, Ng GKH, Wat CKH, But PPH. Pharmacognostic differentiation between *Murraya paniculata* (L.) Jack and *Murraya koenigii* (L.) Spreng. *Pharm Biol* 1986;24(3):167-170.