

Multiplex PCR Assays for Species Discrimination of *Cymbopogon citratus* (DC.) Stapf and *C. nardus* (L.) Rendle, Two Common ‘Serai’ (Lemon Grass) Species in Peninsular Malaysia

(Asai ‘Multiplex PCR’ bagi Membezakan *Cymbopogon citratus* (DC.) Stapf dan *C. nardus* (L.) Rendle, Dua Spesies Serai yang Biasa Diperoleh di Semenanjung Malaysia)

WEI LUN NG, SWEE KEONG YEAP, NUR SYAZANA MOHAMED ABU BAKAR,
WAN NURFATIN WAN MOHD JAAFAR & SOON GUAN TAN*

ABSTRACT

Aromatic grass species Cymbopogon citratus (‘serai biasa/serai makan’) and *C. nardus* (‘serai wangi’) can be commonly found throughout Peninsular Malaysia. *C. citratus* is used in traditional Malaysian cooking and brewed as tea, while *C. nardus* is used in traditional medicine for external application and in insect repellents. Due to similar morphologies, it can be difficult to tell apart the species at times. Based on DNA sequence alignments of three chloroplast DNA intergenic spacer regions, namely *atpB-rbcL*, *trnH-psbA* and *trnL-trnF*, we designed species-specific primers for multiplex PCR assays for rapid species discrimination between *C. citratus* and *C. nardus*. The method described here makes use of simple molecular techniques that are time- and cost-effective for large-scale application. Such an assay will be useful for the quality assurance of food and medicinal products.

Keywords: Food quality assurance; herb; molecular identification; serai makan; serai wangi

ABSTRAK

Spesies rumput aromatik Cymbopogon citratus (‘serai biasa/serai makan’) dan *C. nardus* (‘serai wangi’) biasa diperolehi di sekitar Semenanjung Malaysia. *C. citratus* digunakan dalam masakan tradisi Malaysia dan direbus sebagai teh, manakala *C. nardus* digunakan dalam perubatan tradisi untuk aplikasi luaran dan penghalau serangga. Oleh kerana morfologinya yang sama, kedua-dua spesies ini sukar untuk dibezakan. Berdasarkan penjajaran jujukan DNA daripada tiga ‘intergenic spacer’ DNA kloroplas iaitu *atpB-rbcL*, *trnH-psbA* dan *trnL-trnF*, ‘primer’ khusus telah dihasilkan untuk kaedah ‘multiplex PCR’ bagi membezakan *C. citratus* dan *C. nardus* dengan cepat. Kaedah yang digunakan melibatkan teknik molekul ringkas yang menjimatkan masa dan kos untuk penggunaan berskala besar. Kaedah ini berguna dalam proses penjaminan kualiti produk makanan dan ubat-ubatan.

Kata kunci: Herba; jaminan kualiti makanan; pengesanan secara molekul; serai makan; serai wangi

INTRODUCTION

Cymbopogon is a genus under the Poaceae or the grass family comprising of species that are rapidly growing and possess distinctive aromatic foliage. The species are indigenous in tropical and semi-tropical areas of Asia and are cultivated in other tropical regions of the world (Akhila 2010). In Peninsular Malaysia, two *Cymbopogon* species, *C. citratus* (lemon grass) and *C. nardus* (citronella grass) can be commonly found. Locally known as ‘serai biasa’ or ‘serai makan’ (literally, the common lemongrass or edible lemon grass), *C. citratus* is used as a herb in day-to-day preparation of local Malaysian dishes as well as being brewed as tea; while ‘serai wangi’ (literally, fragrant lemon grass; *C. nardus*) is traditionally used topically (i.e. not ingested), such as in the preparation of bath water meant for women undergoing confinement and as a natural insect repellent (pers. comm. Wei Lun Ng). The essential oils derived from both *C. citratus* and *C. nardus* have been

shown to possess insect repellent properties, although in most cases the essential oil from *C. nardus* seems to be more effective (Maia & Moore 2011). Due to their uses, both species can be found planted around housing areas in Peninsular Malaysia, although *C. citratus* is more frequently spotted and its stalks are also sold in local wet markets (pers. obs. Wei Lun Ng).

Based on our observations in the field, mature bushes of both species have only slight differences: *C. citratus* has leaves that are light green in color, are narrower (~1.4 cm) and shorter (~95 cm) and its bush grows to a height of ~130 cm; compared to *C. nardus* which has leaves that are dark green in colour, are broader (~2.7 cm) and longer (~150 cm) and its bush grows to a height of ~150 cm. Despite these differences, such continuous (quantitative) traits can be variable in plants (Sultan 2000) and the species can be difficult to tell apart when the plants are young or when the leaves are not present (very often only the stalk

is retained when being sold at the wet markets). A DNA marker for species discrimination between *C. citratus* and *C. nardus* will be useful for quality assurance of food and medicinal products, especially when morphological data is not available.

While DNA barcoding by DNA sequencing is accurate and is gaining popularity as a tool for species identification, its cost limits the number of samples to be screened. Here we describe an easy, rapid and inexpensive multiplex PCR assay for molecular species discrimination of *C. citratus* and *C. nardus* through amplification of species-specific DNA fragments based on chloroplast DNA intergenic spacer regions.

MATERIALS AND METHODS

SAMPLING AND DNA EXTRACTION

Three samples each of *C. citratus* and *C. nardus* were obtained from different locations within Peninsular Malaysia (Terengganu: Dungun, Melaka: Ayer Keroh and Negeri Sembilan: Seremban for *C. citratus*; Selangor: Kajang and Serdang and Kedah: Nyak Gam for *C. nardus*;

the exact sampling locations are shown in a map in Figure 1) to maximise the coverage of any genetic variation possibly present in the species. The samples collected comprised of either the leaf or the stalk or both, that were frozen prior to DNA extraction.

Genomic DNA (gDNA) was extracted from 100 mg leaf/stalk material using the cetyl trimethylammonium bromide (CTAB) extraction method. The extracted gDNA was subsequently used as template for PCR amplification.

PCR AMPLIFICATION AND DNA SEQUENCING

Three chloroplast DNA (cpDNA) regions: the *atpB-rbcL* intergenic spacer (IGS), *trnH-psbA* IGS and *trnL-trnF* IGS regions were PCR-amplified from the gDNA of both *C. citratus* and *C. nardus*. The universal primers *atpB*-1 (5'-ACATCKARTACKGGACCAATAA-3') and *rbcL*-1 (5'-AACACCAGCTTTRAATCCAA-3') by Chiang et al. (1998), *trnH*-1 (5'-CGCGCATGGTGGATTCAATCC-3') and *psbA*-1 (5'-GTTATGCATGAACGTAATGCT-3') by Kress et al. (2005), *trnL*-c (5'-CGAAATCGGTAGACGCTACG-3') and *trnF*-f (5'-ATTTGAACTGGTGACACGAG-3') by Taberlet et al. (1991), respectively, were used. PCR

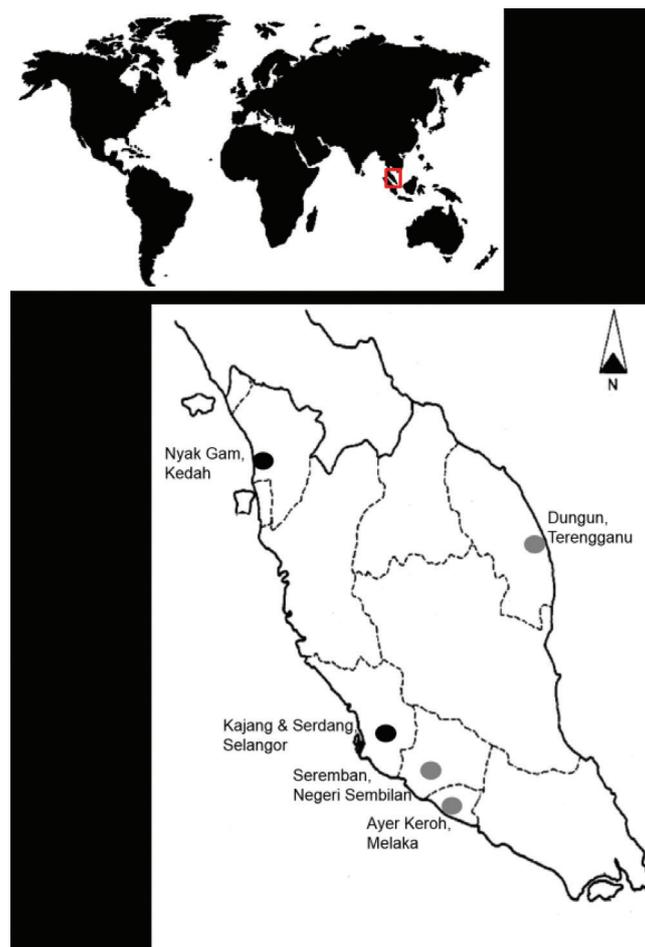


FIGURE 1. Map showing the sampling locations for the three *C. citratus* and three *C. nardus* samples used in this study. Grey circles represent sampling locations for *C. citratus*; black circles represent sampling locations for *C. nardus*

amplifications were performed in 20 μ L reaction mixtures, each containing 30–50 ng of gDNA as template, 1 μ M of each primer and 10 μ L of NEXpro™ e PCR 2 \times master mix (NEX Diagnostics). The PCR reaction profile comprised of an initial denaturation of 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 50°C and 2 min at 72°C and finally an extension step of 7 min at 72°C.

The purified PCR products were used for direct sequencing with the BigDye® Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems) and the products were analysed on an ABI3730xl DNA Analyzer (Applied Biosystems) through services provided by First BASE Laboratories Sdn. Bhd.

DATA ANALYSIS AND PRIMER DESIGN

The cpDNA sequences were edited using the software ATGC ver. 6.0 (GENETYX CORPORATION) and aligned using Clustal W (Thompson et al. 1994) implemented in MEGA 5 (Tamura et al. 2011) and corrected manually.

Based on the alignment of the cpDNA sequences, single nucleotide polymorphisms (SNPs) specific to each of the species were identified. Primers specific to the DNA sequence of *C. nardus* were then designed: the 3' ends of the primers were designed to perfectly match the SNP specific to *C. nardus*. An additional mismatch was introduced at the third nucleotide position from the 3' ends to increase the power of selective amplification during PCR, as in Ng and Szmids (2013).

RESULTS AND DISCUSSION

DNA SEQUENCE ALIGNMENT

Three cpDNA IGS regions were first sequenced from one sample each of *C. citratus* and *C. nardus*. As the *atpB-rbcL* IGS region contained several long mononucleotide repeats which affected the efficiency of DNA sequencing, its sequences were not conclusive and excluded from subsequent analyses. Such conditions are due to the formation of stutter products, a common artifact during PCR amplification of long, repeated sequences (Clarke et al. 2001). DNA sequence alignments were thus performed separately for the remaining two IGS regions. No interspecific site polymorphism was found in the *trnH-psbA* IGS regions, while only one species-specific polymorphic site was found in the *trnL-trnF* IGS region. The *trnL-trnF* IGS region of two more samples of each

species was additionally sequenced and it was confirmed that the polymorphism was species-specific.

The DNA sequences for both species at the *trnL-trnF* (893bp) and *trnH-psbA* (573bp) IGS regions obtained in this study have been deposited in GenBank with accession numbers KT274018–KT274021.

PRIMER DESIGN AND MULTIPLEX PCR

Out of the three IGS regions, species-specific primers were successfully designed only for the *trnL-trnF* IGS region. Two primers, in opposite directions, were designed with their 3' ends anchoring at the polymorphic site specific for *C. nardus* (Figure 2). Multiplex PCR was conducted using any one of the two designed primers together with the *trnL-c* and *trnF-f* primers (a total of three primers per PCR reaction) on *C. citratus* and *C. nardus* samples. The two sets of primers (sets A and B) for multiplex PCR are shown in Table 1. PCR amplifications were performed in 10 μ L reaction mixtures, each containing 30–50 ng of gDNA as template, 0.5 μ M of each primer and 5 μ L of NEXpro™ e PCR 2 \times master mix (NEX Diagnostics). The PCR reaction profile comprised of an initial denaturation of 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 50 or 55°C and 2 min at 72°C and finally an extension step of 7 min at 72°C. PCR amplicons were analysed by electrophoresis on 1.0% (weight/volume) agarose gel, stained with ethidium bromide and viewed under UV illumination.

The use of either set of primers yielded the intended banding patterns on the agarose gel (i.e. one band for *C. citratus* and two bands for *C. nardus*; their corresponding band sizes are listed in Table 1). The primers were tested on 8 other samples each of *C. citratus* and *C. nardus* collected from random sites in Peninsular Malaysia and the results were consistent in all cases. As shown in Figure 3, the targeted species-specific bands were clear and can be easily sized on a 1.0% agarose gel, despite of some amount smearing/unspecific amplification that did not affect the quality/visualization of the target bands.

Simple multiplex PCR assays have been developed for the reliable identification of a myriad of species, including microbial (Settanni & Corsetti 2006), animal (Fajardo et al. 2010; Lin & Hwang 2008) and plant species (James et al. 2003; Ng & Szmids 2013), the identification of which through morphology or various tests were initially complicated tasks. With *C. citratus* and *C. nardus* being commonly used in the food and natural product industries,

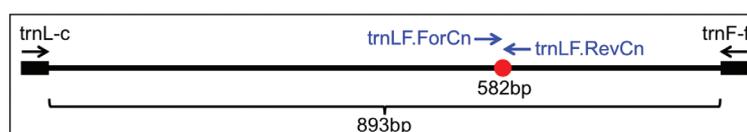


FIGURE 2. A schematic diagram showing the positions and directions of the primers used in the multiplex PCR assays. The red dot shows the location of the species-specific site mutation between *C. citratus* and *C. nardus* at the amplified *trnL-trnF* IGS region

TABLE 1. PCR primers used in the multiplex assays developed in this study

Primer set	Primer sequences	Target species (no. of bands; approx. band size(s))
A	trnL-c 5'-CGAAATCGGTAGACGCTACG-3'	<i>C. citratus</i> (1 band; ~950bp)
	trnF-f 5'-ATTTGAACTGGTGACACGAG-3'	<i>C. nardus</i> (2 bands; ~350bp and ~950bp)
	trnLF.ForCn 5'-GGGTTTAAGATTCACTAGCT C <u>T</u> C-3'	
B	trnL-c 5'-CGAAATCGGTAGACGCTACG-3'	<i>C. citratus</i> (1 band; ~950bp)
	trnF-f 5'-ATTTGAACTGGTGACACGAG-3'	<i>C. nardus</i> (2 bands; ~630bp and ~950bp)
	trnLF.RevCn 5'-CCTTTGTGAAAGAGTAGAAT C <u>A</u> G-3'	

For primers trnLF.ForCn and trnLF.RevCn, bolded letters represent species-specific SNP matches; underlined letters represent introduced mismatches

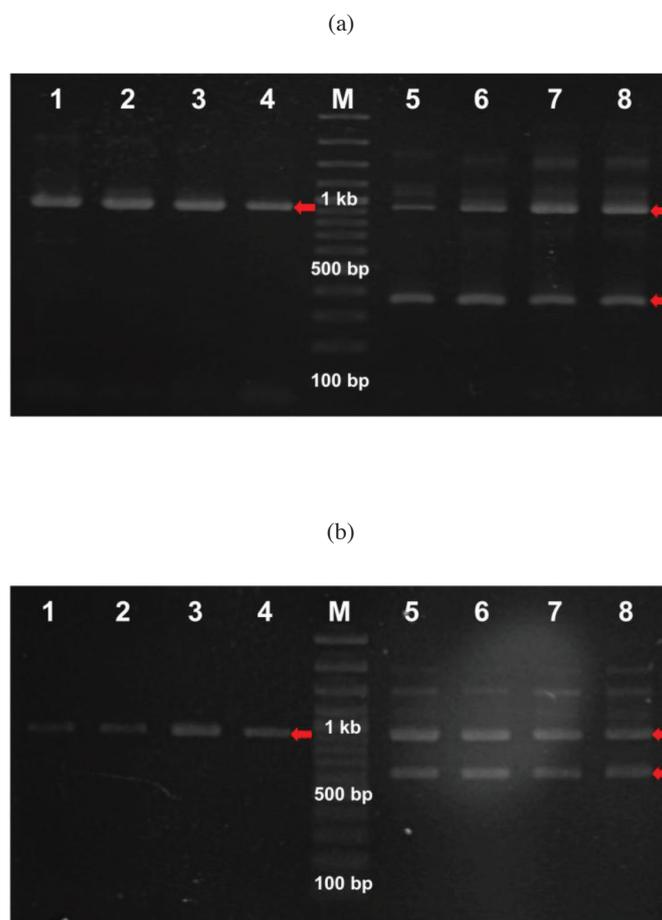


FIGURE 3. Multiplex PCR amplification results of *C. citratus* and *C. nardus* samples using (a) primer set A and (b) primer set B. Lane M = 100 bp DNA ladder; lanes 1-4 = *C. citratus*; lanes 5-8 = *C. nardus*. Red arrows show the expected bands

we believe that the method described in this study will provide a way for increased product quality assurance through molecular species identification.

ACKNOWLEDGEMENTS

The authors thank the anonymous reviewers for providing helpful comments on earlier drafts of the manuscript.

REFERENCES

- Akhila, A. 2010. *Essential Oil-Bearing Grasses: The genus Cymbopogon*. Florida: CRC Press.
- Chiang, T.Y., Schaal, B.A. & Peng, C.I. 1998. Universal primers for amplification and sequencing a noncoding spacer between the *atpB* and *rbcL* genes of chloroplast DNA. *Bot. Bull. Acad. Sin.* 39: 245-250.
- Clarke, L.A., Rebelo, C.S., Goncalves, J., Boavida, M.G. & Jordan, P. 2001. PCR amplification introduces errors into mononucleotide and dinucleotide repeat sequences. *J. Clin. Pathol.: Mol. Pathol.* 54: 351-353.
- Fajardo, V., Gonzalez, I., Rojas, M., Garcia, T. & Martin, R. 2010. A review of current PCR-based methodologies for the authentication of meats from game animal species. *Trends in Food Sci. & Tech.* 21(8): 408-421.
- James, D., Schmidt, A., Wall, E., Green, M. & Masri, S. 2003. Reliable detection and identification of genetically modified maize, soybean, and canola by multiplex PCR analysis. *J. Agric. Food Chem.* 51(20): 5829-5834.
- Kress, W.J., Wurdack, K.J., Zimmer, E.A., Weigt, L.A. & Janzen, D.H. 2005. Use of DNA barcodes to identify flowering plants. *PNAS* 102(23): 8369-8374.
- Lin, W.F. & Hwang, D.F. 2007. A multiplex PCR assay for species identification of raw and cooked bonito. *Food Control* 19(9): 879-885.
- Maia, M.F. & Moore, S.J. 2011. Plant-based insect repellents: a review of their efficacy, development and testing. *Malaria Journal* 10(Sup 1): S11.
- Ng, W.L. & Szmidt, A.E. 2013. A simple and inexpensive molecular assay for species identification of Indo-West Pacific *Rhizophora* mangroves for conservation and management. *Conservation Genet. Resour.* 5: 1059-1061.
- Settanni, L. & Corsetti, A. 2006. The use of multiplex PCR to detect and differentiate food- and beverage-associated microorganisms: A review. *J. Microbiological Methods* 69(1): 1-22.
- Sultan, S.E. 2000. Phenotypic plasticity for plant development, function and life history. *Trends in Plant Sci.* 5(12): 537-542.
- Taberlet, P., Gielly, L., Pautou, G. & Bouvet, J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol.* 17: 1105-1109.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28: 2731-2739.
- Thompson, J.D., Higgins, D.G. & Gibson, T.J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680.
- Wei Lun Ng, NurSyazana Mohamed Abu Bakar,
Wan Nurfatim Wan Mohd Jaafar & Soon Guan Tan*
Department of Cell and Molecular Biology
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
43400 Serdang, Selangor Darul Ehsan
Malaysia
- Swee Keong Yeap
Institut Biosains, Universiti Putra Malaysia
43400 Serdang, Selangor Darul Ehsan
Malaysia
- *Corresponding author; email: sgtan@upm.edu.my
- Received: 30 April 2015
Accepted: 28 September 2015