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 Specie 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 wang’) 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 as 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 wang

ABSTRAK


Kata kunci: Herba; jaminan kualiti makanan; pengesahan secara molekul; serai makan; serai wang

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 wang’ (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 (~130 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'-CCCGCATGTTGGATTCACAATCC-3') and *psbA-1* (5'-GTGTGCTGACGTAATGCT-3') by Kress et al. (2005), *trnL-c* (5'-CGAAATCGGTAGACGCTACG-3') and *trnF-f* (5'-ATTGAACTGGTGACACGAG-3') by Taberlet et al. (1991), respectively, were used. PCR

![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*.](image)
amplifications were performed in 20 μL reaction mixtures, each containing 30-50 ng of gDNA as template, 1 μM of each primer and 10 μL of NEXpro™ PCR 2× 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 Szmidt (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 μL reaction mixtures, each containing 30-50 ng of gDNA as template, 0.5 μM of each primer and 5 μL of NEXpro™ e PCR 2× 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 & Szmidt 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](image-url)
### TABLE 1. PCR primers used in the multiplex assays developed in this study

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Primer sequences</th>
<th>Target species</th>
<th>(no. of bands; approx. band size(s))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>trnL-c 5’-CGAAATCGGTAGACGCTACG-3’</td>
<td>C. citratus</td>
<td>(1 band; ~950bp)</td>
</tr>
<tr>
<td></td>
<td>trnF-f 5’-ATTTGAACTGGTGACACGAG-3’</td>
<td>C. nardus</td>
<td>(2 bands; ~350bp and ~950bp)</td>
</tr>
<tr>
<td></td>
<td>trnLF.ForCn 5’-GGGTTTAAGATTCACTAGCCTGTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>trnL-c 5’-CGAAATCGGTAGACGCTACG-3’</td>
<td>C. citratus</td>
<td>(1 band; ~950bp)</td>
</tr>
<tr>
<td></td>
<td>trnF-f 5’-ATTTGAACTGGTGACACGAG-3’</td>
<td>C. nardus</td>
<td>(2 bands; ~630bp and ~950bp)</td>
</tr>
<tr>
<td></td>
<td>trnLF.RevCn 5’-CCTTTGTGAAAGATAGAATCAGGACAG-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

![Figure 3a](image1.png)
![Figure 3b](image2.png)

**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.

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Wei Lun Ng, NurSyazana Mohamed Abu Bakar, Wan Nurfatin 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

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