Gene 573 (2015) 84–90

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Authenticity analyses of *Phyllanthus amarus* using barcoding coupled with HRM analysis to control its quality for medicinal plant product

Kittisak Buddhachat [a], Maslin Osathanunkul [a], Panagiotis Madesis [b], Siriwadee Chomdej [a,d,*], Siriwan Ongchai [c]

[a] Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
[b] Institute of Applied Biosciences, Centre for Research & Technology Hellas (CERTH), Thessaloniki, Greece
[c] Thailand Excellence Center for Tissue Engineering and Stem Cells, Department of Biochemistry and Center of Excellence for Innovation in Chemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
[d] Materials Science Research Center, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

ARTICLE INFO

Article history:
Received 6 March 2015
Received in revised form 1 July 2015
Accepted 10 July 2015
Available online 16 July 2015

Keywords:
DNA barcoding
Phyllanthus amarus
Medicinal plant
HRM
*rbc*L
*trn*L

ABSTRACT

The *Phyllanthus* genus, a plant used in traditional Thai medicine, has according to several pharmacopeias hepatoprotective properties. Not only is the anatomical morphology of these species relatively similar but they also share the Thai common names Look-Tai-Bai (ลูกใต้ใบ) and Yah-Tai-Bai (หญ้าใต้ใบ), which might cause confusion for laypersons. This study attempted to develop a method for accurate identification of *Phyllanthus* species, especially *Phyllanthus amarus*, and to detect contaminants in *P. amarus* products by using DNA barcoding coupled with high resolution melting (HRM) analysis (bar-HRM). Two plastid loci (*rbc*L and *trn*L) were chosen for DNA barcoding to generate a suitable primer for distinguishing *Phyllanthus* species by HRM analysis. The five species of *Phyllanthus* were subjected to amplification for testing the specificity and discrimination power of the designed primers derived from *rbc*L and *trn*L regions. Sensitivity of the method (DNA barcoding conjugated with HRM) to detect adulterant in *P. amarus* samples was evaluated. The commercial *P. amarus* products obtained from a local market were authenticated. The primer pair derived from *trn*L DNA barcoding (*Phylltrn*L) had more specificity and power of discrimination for *Phyllanthus* species than that derived from *rbc*L DNA barcoding (*Phyllrbc*L). The result showed that T_m of *P. amarus*, *Phyllanthus urinaria*, *Phyllanthus debilis*, *Phyllanthus airy-shawii*, and *Phyllanthus virgatus* was 74.3 ± 0.08, 73.04 ± 0.07, 73.36 ± 0.05, 72.21 ± 0.06, 72.77 ± 0.15 °C, respectively. This method proved to be a very sensitive tool that can be used for rapid detection of contamination as low as 1% of other *Phyllanthus* species in *P. amarus* admixtures. All commercial products of *P. amarus* obtained from a local market in Thailand were found to contain pure raw materials of *P. amarus* without any substitution or contamination. Our results indicated that the use of DNA barcoding coupled with HRM was an efficient molecular tool for correct species identification. This molecular tool provides a noteworthy benefit for quality control of medicinal plants and industry plants for pharmacological prospects.

1. Introduction

Phyllanthus genus is one of the most diverse groups in the plant kingdom with 833 species (Chaudhary and Rao, 2002). Hepatoprotectivity is a dominant biological property of *Phyllanthus* (*Phyllanthus amarus*, *Phyllanthus niruri*, *Phyllanthus urinaria*, *Phyllanthus emblica*, *Phyllantus debilis* and *Phyllanthus maderaspatensis*). Most extracts from *Phylllanthus* genus contain biologically active compounds responsible for liver protection from any toxic substances. Despite the similar activity in various *Phylllanthus* extracts, the chemical constituents in each species of *Phylllanthus* are unlike. Khatoon et al. (2006) and Tripathi et al. (2006) reported large amounts of phyllanthin and hypophyllanthin detected especially in *P. amarus*, whereas small quantities might be found in some species such as *P. urinaria* and *Phyllanthus virgatus*. Phyllanthin is a hepatoprotective compound that enables protection against ethanol-induced oxidative stress causing liver cell damage in rats through its antioxidant activity against superoxide dismutase (SOD) and glutathione reductase (GR) (Chirdchupunseree and Pramyothin, 2010). Additionally, phyllanthin and hypophyllanthin could modulate the vascular tension via the endothelium-independent mechanism. The modulating effects of both compounds are possibly

Abbreviations: *atp*B, beta subunit of ATP synthase; *mat*K, maturase K; *ndh*F, NADH dehydrogenase 5; *rbc*L, ribulose-bisphosphate carboxylase; HRM, high resolution melting analysis.

* Corresponding author at: Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail address: siriwadee.submission@gmail.com (S. Chomdej).

http://dx.doi.org/10.1016/j.gene.2015.07.046
0378-1119/

involved in blocking Ca^{2+} entry into vascular smooth muscle cells and inhibiting PE-mediated Ca^{2+} release from sarcoplasmic reticulum (Inchoo et al., 2011). In addition, the extract of *P. amarus* is capable of inhibiting the activity of alpha-amylase, which is a target enzyme for diabetes treatment (Ali et al., 2006). Kiemer et al. (2003) demonstrated that the standardized extract of *P. amarus*, as an anti-inflammatory agent, enabled the reduction of iNOS, COX-2 production in RAW264.7 via suppressing the NF-κb pathway and could alleviate TNF-α synthesis in rats.

P. amarus is a herbaceous plant that can be found throughout Thailand and is known in Thai by the following vernacular names "Look Tai Bai" (ลูกใต้ใบ) meaning "seeds under leaf". However, several other species of *Phyllanthus* genus also go by the names "Look Tai Bai" and/or "Yah Tai Bai", including *P. urinaria*, *P. debilis*, *P. virgatus* and *Phyllanthus fraternus* due to their similar macromorphology, i.e. the presence of seeds under the leaves. Therefore, plant material of *P. amarus* picked by a layperson may result in the collection of additional undesired species and thereby causing the end product to be of inferior quality. As the plant material is processed to a powder form e.g. capsules or tea, the plant species cannot be identified morphologically, but since genetic material still remains in the product samples, accurate plant species identification can be achieved through DNA barcoding.

A method using short orthologous DNA sequences, known as "DNA barcodes", has been successfully applied for identifying plant species, especially loci in the plastid genome such as *rbc*L, *mat*K, *trn*H-*psb*A, *trn*L-F, *ndh*F, and *atp*B loci as well as the internal transcribed spacer (ITS) of the nuclear ribosomal cistron (18S–5.8S–26.S) (Hollingsworth et al., 2011; Chen et al., 2010; Kress et al., 2005). These loci have been selected as the standard regions for taxonomic identification of plants species. CBOL Plant Working Group (2009) suggested that the combination of *rbc*L and matK provides a universal framework for routine use for species identification of terrestrial plants. *trn*L loci have also been proposed for species identification. Although *trn*L loci provide a relatively low resolution, its primer is highly conserved and the amplification system is very robust (Taberlet et al., 2007). Furthermore, the combined use of *rbc*L and *trn*L as a two-locus cpDNA barcode has shown a high potential for identifying NW-European fern species (de Groot et al., 2011). The huge online digital library of DNA barcoding serves as the information to be searchable for unidentified samples from nature or the market using one or a few DNA barcoding loci (Kress and Erickson, 2008).

High resolution melting (HRM) analysis has become one of the most important techniques for mutation and methylation analyses. Therefore, HRM has been widely applied for genotype scanning that does not require probe. SYBR green, a homogenously intercalating agent for binding duplex DNA, is a widespread fluorescent dye to detect mutation by HRM. The amplicons obtained after the amplification process are detached from double-strand DNA to single-strand DNA, gaining melting temperature (T_m). The T_m value of a particular PCR product depends on sequence length, GC content complementarity and nearest neighbor (Reed and Wittwer, 2004). The use of HRM coupled with DNA barcoding, SNP markers and microsatellites enables taxonomical identification and detection of contaminants in food products for quality assurance (Ganopoulos et al., 2012; Madesis et al., 2012; Ganopoulos et al., 2011). HRM coupled with DNA barcoding, *trn*L loci, was applied to determine the presence of contaminants in "*Fava Santorinis P.D.O.*" commercial food products (Ganopoulos et al., 2012). Moreover, HRM analysis has been used for testing juice, cherry and meat of contaminants (Faria et al., 2013; Sakaridis et al., 2013; Ganopoulos et al., 2011). Until now, it has never been applied as a method for species identification of medicinal plants.

In order to assure of a dry or post-processed plant material studied whether it is *P. amarus* or according to its label on the products, we provided a new, fast and accurate method to identify *Phyllanthus* spp. by DNA barcoding coupled with HRM analysis.

2. Materials and methods

2.1. Plant materials and DNA isolation

Phyllanthus species (*P. amarus*, *P. urinaria*, *P. airy-shawii*, *P. debilis* and *P. virgatus*) used in this study were collected around Chiang Mai University, Chiang Mai, Thailand and identified through a key from Flora of Thailand Euphorbiaceae (http://www.nationaalherbarium.nl/ThaiEuph/). As these plant species have a similar morphology resulting in the same common Thai name, "Look Tai Bai" or "Yai Tai Bai", and are often found in the same or adjoining areas, the identification of the plant material was done by an expert (Taxonomist). The different medicinal plant genera (Table S1), were acquired from the Botanical Garden, Faculty of Pharmacy, Chiang Mai University. In addition, six commercial products of *Phyllanthus* species were purchased from a local market in Thailand. The plant material was ground into powder form after freezing with liquid nitrogen. The starting 100 mg of fine powder was used for DNA extraction using *Nucleospin Plant® II* (Macherey-Nagel, Germany) and following the instructions of the manufacturer; the DNA was stored at −20 °C for further use.

2.2. PCR amplification, sequencing and primer design

The amplifications of DNA barcoding region in chloroplast, *rbc*L and *trn*L loci, were performed in a total volume of 25 μl, containing 1 μl of 50 ng DNA template with primer sets (*rbc*L_F; ATGTCACCACAAACAG AGACTAAAGC, *rbc*L_R; GTAAAATCAAGTCCACCRCG and *trn*L_F; GGGG ATAGAGGGACTTGAAC, *trn*L_R; CGAAATCGGTAGACGCTACG) 1 U of Taq DNA polymerase (*RBC*, Taiwan), 200 mM of each dNTP, 0.2 μM of each primer, and 1× PCR buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 0.1% Triton X-100 and 2 mM of $MgCl_2$). PCR amplification was performed using *MyCycler* (BioRAD, Applied Biosystems, USA). An initial temperature at 94 °C for 5 min was used, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. The final extension was done at 72 °C for 7 min, followed by a hold step at 4 °C. The amplified DNA was subjected to electrophoresis in a 1.5% agarose gel (120 V, 30 min) pre-stained with ethidium-bromide, and viewed under ultra-violet (UV) light by a UV transmitter (BioRAD, Applied Biosystems, USA). PCR products were directly sequenced at 1st BASE company (Malaysia). The sequences obtained from *rbc*L and *trn*L loci were deposited in *Genbank* (Table S1) aligned using *SeqMan* program to generate primer sets that are applicable for barcoding with HRM method to identify each *Phyllanthus* species. The primer sets should yield products not exceeding 200 bp and there are variations of nucleotides within the PCR products. The primer sets designed and used in this study are *Phyllrbc*L_F; ACGATCAAGACTGGTA AGTCCGT, *Phyllrbc*L_R; CTGAGGAAGCGGGGGCTGCGGTAG, *Phylltrn*L_F; TTGAGATAGTATTTCAGTACCTA, and *Phylltrn*L_F; CTTCCATCAAAACTCC AGAAAAG.

2.3. Determination of primer specificity

To evaluate the specificity of the primer sets for bar-HRM obtained from *rbc*L and *trn*L loci, the annealing temperature (T_a) optimization of each primer set was carried out using gradient PCR ranging from 50 °C to 60 °C. The results showed that all T_a can produce 88-amplicon and 99-amplicon which was the expected PCR product for *rbc*L and *trn*L loci, respectively, and there is no non-specific band (data not show). Therefore, we selected the annealing temperature at 60 °C for further PCR amplification or HRM method. Subsequently, DNAs isolated from *Phylllanthus* species and the other medicinal plant species were used as template for primer specificity test with the reaction mixture and PCR protocol as described above.