DNA Barcoding for Authentication of *Orthosiphon stamineus* Herbal Medicinal Product Using *ITS2* Nuclear Marker Originating from Malaysia

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Abstract. *Orthosiphon stamineus* (Misai Kucing) has become the focus of commercialization in Malaysia due to its potential health treatment benefits that can attract huge consumers' demand. Although the quality of herbal medicinal product (HMP) remains a major concern for consumers due to its high adulteration rate, there is insufficient testing done to determine whether the herb species used in HMP are as claimed in their ingredients. HMP authentication can be done by using a DNA barcoding approach which is species-specific. In this work, the first nuclear *ITS2* barcode (279bp) from the fresh plant of *O. stamineus* (MKP) originating from Malaysia was successfully generated and deposited in Genbank (MT251295.1). The efficiency of the novel barcode in *O. stamineus* HMP authentication was tested in two samples, tea (MKT) and capsule (MKC) based on BLAST and NJ-tree analysis. The success of applying DNA barcoding by using *ITS2* barcode generated in this work for HMP authentication makes it possible to complement conventional methods for *O. stamineus* HMP quality assurance testing and authentication in Malaysian herbal industry.

1. Data Description

Currently, herbal medicinal products (HMP) made from *Orthosiphon stamineus* have gained an increase in popularity among consumers due to its well-known health benefits provided by its high polyphenol content that is dominated by rosmarinic acid and sinensetin, largely found in *O. stamineus* leaf [1], causing it to become the focus of commercialization of the Malaysian government. However, the authenticity of the HMP has become the greatest concern for consumers due to unscrupulous commercial practice such as adulteration and herb species misidentification during the HMP production [2]. Furthermore, there are limitations of using conventional approaches for HMP authentication, for instance, the phytochemical analysis that is not species-specific and the highly processed HMPs caused herbs species identification by taxonomists to be impossible [3]. Meanwhile, in spite of the prominent role of the traditional medicine system and high adulteration rate of HMP identified, there is insufficient testing conducted for herbs species identification in HMPs by DNA-based method, for instance, DNA barcoding [4]. To date, only plastid DNA barcodes such as *rbcL* and *trnH-psbA* had been employed for the authentication of *O. stamineus* HMP. However, HMP authentication using this two-tiered barcode approach has several drawbacks. For instance, low DNA barcode recovery of only 73.4% due to high DNA degradation in HMP and higher cost incurred due to inclusion of more barcodes [5]. Therefore, nuclear barcodes such as *ITS2* which is being identified as the most suitable barcode to be used in HMP authentication due to its several valuable characteristics. The availability of conserved regions for

universal primer designing purpose, fewer amplification and sequencing problem due to its short sequence length, and high interspecific divergence that allow sufficient sequence variability for closely related species distinguishing purpose [6][7], making *ITS2* a more suitable barcode to be used in HMP authentication. Despite the fact that *ITS2* has been identified as the most suitable barcode to be used in HMP authentication, there is a lack of reference *O. stamineus* nuclear barcode sampled from Malaysia either in Genbank or BOLD database.

The BLASTn analysis showed that the *ITS2* barcode (279bp) of *O. stamineus* leaves (MKP) obtained in this work had a very high percentage identity (97% to 100%) to other *O. stamineus* (synonym *O. aristatus*) sequence in Genbank. The barcode resulted in 100% identity with *O. stamineus ITS2* (FJ593403.1) originating from China [8]; JF301407.1 from unknown sample origin [9], and AY506663.1 sampled from old world tropical [10], a term normally used in the west referring to Africa, Asia and Europe. However, for the percentage identity of *ITS2* of *MKP* with other species from the same genus such as *Orthosiphon wulfenioides*, only 93.6% was shown, and did not meet the minimum BLAST cut-off of 97% for a top match [11] [12]. Phylogenetic tree was constructed using a neighbour-joining (NJ) method for the trimmed sequence from MKP with several high scoring BLASTn hits as shown in Figure 1. *Perilla citriodora* (accession no. KT220699.1) was used as an outgroup in this NJ-phylogenetic tree. From the phylogenetic tree, it indicates that *ITS2* of MKP formed a strongly supported monophyletic clade with other *O. stamineus* (aka *O. aristatus*). Hence from the BLAST and NJ-phylogenetic analysis, *ITS2* barcode obtained from MKP in this work is confirmed as *O. stamineus* besides showing this barcode generated has high discriminatory power, whereby it allows the discrimination between species in the same genus, for instance, the discrimination between *O. stamineus* and *O. wulfenoides* in this case. The verified *ITS2* barcode generated in this work is now available in the Genbank database with the accession number of MT251295.1. Notably, this *ITS2* barcode is also the first *O. stamineus ITS2* barcode originating from Malaysia.

The application of *O. stamineus ITS2* generated from this work (MT251295.1) as internal reference barcode for authentication of Malaysian *O. stamineus*-based HMP, was tested in tea and capsule samples, referred as MKT and MKC respectively. The HMPs tested were declared to only contain *O. stamineus* on the packaging. Megablast and NJ-phylogenetic tree analysis (Figure 1) confirmed that *ITS2* amplicons from MKT and MKC, both were highly identical (99-100%) with 100% query coverage to the internal reference barcode (MT251295.1) compared to other *O. stamineus* sequences available in Genbank. This suggests that the use of this internal reference barcode originating from Malaysia does facilitate HMP authentication and allows the identification of species to be done more accurately. Thus, the success of applying DNA barcoding using *ITS2* barcode generated in this work for HMP authentication in our study makes it possible to utilize this *ITS2* barcode for DNA barcoding to complement conventional methods for *O. stamineus* HMP quality assurance testing and authentication in the Malaysian herbal industry.

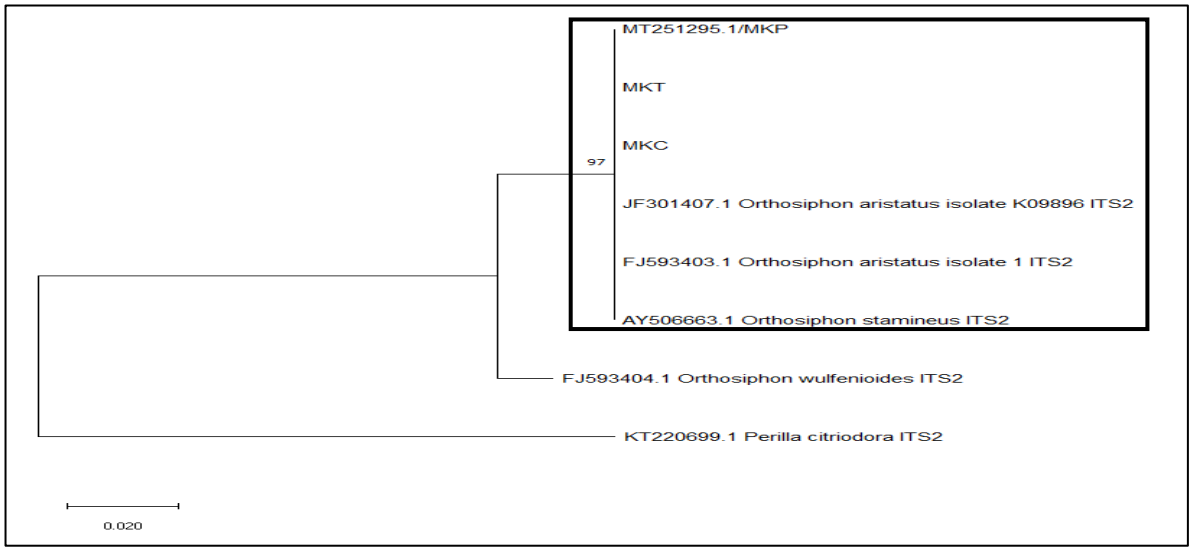

Figure 1. Neighbour-joining tree of *ITS2* from *O. stamineus* HMPs (MKT and MKC) compared to *ITS2 O. stamineus* barcode sequences established in this study (MT251295.1/MKP) and other sequences retrieved from the GenBank. Black box showed the clade cluster of HMPs containing *O. stamineus* with the internal reference barcode generated (MT251295.1/MKP); JF301407.1 *Orthosiphon aristatus* isolate K09896; FJ593403.1 *Orthosiphon aristatus* isolate 1 and AY506663.1 *Orthosiphon stamineus*.

2. Experimental Design, Materials and Methods

2.1 Sample collection, gDNA extraction and PCR

Orthosiphon stamineus plant was bought from a local nursery and sent to Forest Research Institute Malaysia (FRIM) for verification (Voucher no: PID 250817-18). The monoherbal *O. stamineus* herbal medicinal products (HMP) in the form of tea sachets (MKT) and capsule (MKC) were purchased online. The genomic DNA extraction from *O. stamineus* fresh leaves (MKP) and its HMPs were carried out by using NucleoSpin® Plant II kit. The gDNA extracted from MKP, MKT and MKC were subjected to quantitative analysis by Nanodrop spectrophotometer and qualitative analysis by agarose gel electrophoresis before being used for *ITS2* barcode amplification by polymerase chain reaction (PCR). The sequence for *ITS2* forward and reverse primer used in this study were GGGGCGGATATTGGCCTCCCCTTGC and GACGCTTCTCCAGACTACAAT respectively [13]. PCR was carried out by using Eppendorf MasterCycler Nexus Gradient (BioRad) in a 25 µL reaction volume by using standard PCR profile with annealing temperature of 56℃, for 30 cycles. The reagent needed for PCR included 5X Green GoTaq Flexi Buffer, 10 µM *ITS2* forward primer, 10 µM *ITS2* reverse primer, 10 mM dNTPs mix (Promega), 25mM $MgCl_2$, sterile ultrapure water, GoTaq DNA Polymerase (1.25 units/µl) (Promega) and DNA template. The PCR products obtained from MKP, MKT and MKC were sent for direct sequencing by forward primer (Apical Scientific Sdn Bhd).

2.2 *Sequence analysis and validation*

DNA sequences of MKP obtained from sequencing results were first visualized and edited by BioEdit software to trim out the noise at its beginning and end part. The verification of this trimmed sequence was done by using BLASTn, followed by Neighbour-joining (NJ-) phylogenetic tree construction using MEGA X by using the trimmed sequenced from MKP with several high scoring BLASTn hits in order to evaluate the robustness of the inferred tree by 1000 bootstrap re-samplings to obtain node support. From the phylogenetic tree generated, clade with a bootstrap value in the range of 50% to 70% is generally considered as moderately supported true clade while clade with a bootstrap value higher than

70% is considered as strongly supported true clade [14]. The validated *ITS2* barcode generated from MKP was submitted to Genbank after validation by BLASTn and phylogenetic tree analysis.

2.3 *Use of internal reference barcode generated for monoherbal HMP with O. stamineus as its declared ingredient*

The validated *ITS2 O. stamineus* (generated from MKP) in this study was used as internal reference barcode for HMPs (MKT and MKC) authentication by using BLASTn and NJ-phylogenetic tree analysis in order to evaluate the efficiency of this barcode in *O. staminues* HMP authentication by DNA barcoding.

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