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# Species Identification Based on trnH-psbA and ITS2 Genes and Analysis of Mineral Nutrients of Selected Medicinal Plants from Malaysia

Nur-Aqidah A.Aziz[1], Mardiana Idayu Ahmad[1], Darlina Md Naim[2,*]

[1]*Environmental Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 Pulau Pinang, Malaysia.*

[2]*School of Biological Sciences, Universiti Sains Malaysia, 11800 Pulau Pinang, Malaysia.*

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**Abstract**

Accurate plant identification and screening of the elements in medicinal plants have become increasingly important. Some elements can become toxic when existing at high concentrations. The present study presents a molecular identification method to discriminate plant species and assess the mineral nutrients in four selected medicinal plants widely used in traditional healing practices in Malaysia. Medicinal plants were sampled from a secondary forest in Northern Peninsular of Malaysia. Species discrimination was conducted using phylogenetic inference and BLAST method on two target genes, trnH-psbA and ITS2 while the concentration levels of the mineral in terms of macro and micro nutrients (Ca, Fe, Mg, Mn, Zn, Cu ) were determined using ICP-OES spectroscopy in the leaf samples. The present study revealed that the DNA identification method has successfully discriminated all samples to species level and that the trnH-psbA is the best marker for identification. The concentration levels of mineral nutrients ranged from 0.01 to 5.76 mg/kg. within the safety range as recommended by the World Health Organization (WHO). Results of the present study provide important data on the DNA barcoding of medicinal plants and assessment of mineral nutrients, which can be useful for providing scientific information on dietary supplements based on authentic medicinal plants and products.

**Keywords**: DNA barcoding, trnH-psbA, ITS, micronutrient, macronutrient, medicinal plants, ICP-OES.

## 1. Introduction

Existing taxonomic identification of plants group is mainly morphological based. However, there are limitations to relying primarily on morphology when attempting to identify plants species during various stages of their development or when examining fragmentary or processed remains. It has been recognized that rapidly evolving mitochondrial genes, punctuated with highly conserved regions can be recovered via Polymerase Chain Reaction (Mullis, 1990). Folmer *et al.* (1994) and Hebert *et al.* (2003) demonstrated that the 5′end region of cytochrome c oxidase sub unit I (COI) is highly appropriate for discriminating between closely related species across diverse phyla in the animal kingdom, establishing it as the "DNA barcode" locus for broadly identifying animals (Ward *et al.*, 2005). However, the use of COI as a universal plant barcode does not indicate any successful story due to the generally low rate of nucleotide substitution in plants mitochondrial genomes (Hollingsworth *et al.*, 2011). Additionally, the structure of mitochondrial genome in plants has changed rapidly, thus the existence of a universal intergenic spacer at the species level will be precluded (Kress *et al.*, 2005). The Internal Transcribed Spacer (ITS) region of the nuclear ribosomal cistron is the most commonly sequenced locus for plant molecular systematic investigations at the species level (Kress *et al.*, 2005). Several chloroplast gene regions, for example, maturase K (matK) and ribulose 1,5-biphosphate carboxylase /oxygenase large sub unit (rbcL) were also widely used and considered as a core barcodes for plants (Schori and Showalter, 2011).

The important roles of medicinal plants in traditional healing systems have been documented in the literature, and it was found that most of the developing world continues to rely on this for primary health care and home remedies. In Malaysia, medicinal plants, in the form of packaged herbal prepared and manufactured by small and medium-sized industry, as well as pharmaceutical industry, are widely used (Ahmad and Othman, 2015). Consequently, Malaysian Government has chosen herbs industry as the first Entry Point Project (EPP1) for the nation's Agriculture New key Economic Area with the aim to produce

* Corresponding author. e-mail: darlinamdn@usm.my.

high-value products amounting MYR 2.2 billion of the Gross National Income (Ahmad and Othman, 2015). Nowadays, in view of scientific interest, consumer demands promote the development of dietary supplements and new drugs based on medicinal plants. The use of these medicinal plant-based products is rapidly spreading in industrialised health care sector of the 21st century (Abe *et al.*, 2013; Kayani *et al.*, 2014; Siew *et al.*, 2014). Although this has been proven to improve the economy of a country, it has raised an alarming concern related to the purity of the raw plants material used in a preparation of the products. There were opportunities for substitution or adulteration of the raw ingredients of the herbal products due to misidentification of plants, lack of cultivation and the long supply chain from harvesting site to market (Schori and Showalter, 2011). Using barcoding technique, the raw material used to produce herbal products can be ascertained because the substitution within certain plant families (especially Apiaceae and Solanaceae) would give a very bad effect and could be fatal. Therefore, the accurate identification of medicinal plants in relation to their purity and quality as well as a safe application has become increasingly important (Pang and Chen, 2014).

Apart from the problems that will arise due to misidentification of plants, herbal products can be contaminated during growth, development and processing influenced by their environmental factors (Barthwal *et al.*, 2008). The medicinal values of these plants lie in their chemical substances in terms of mineral nutrients and metallic elements that are involved in physiological processes and are important for the proper functioning and maintaining good health in the human body (Gupta *et al*., 2010; Subramanian *et al.*, 2012a). These elements play a pivotal role in biochemical processes and enzyme systems in the human organism even at threshold levels (Mahmood *et al.*, 2013). For instance, calcium (Ca), chromium (Cr), copper (Cu), magnesium (Mg) and zinc (Zn) play important roles in neurochemical transmission and serve as elements of biological molecules in various metabolic processes (Okatch *et al.*, 2012).

Although these elements are often essential for living organism, they become toxic when present at high concentrations (Okem *et al.*, 2014). Street (2012) stated that the amount of mineral nutrients and heavy metals in medicinal plants are known to pose potential threats to animals and human beings that consume them or their derived products. For instance, some elements, such as iron (Fe) and magnesium (Mg), can cause ill effects when consumed at higher concentration levels (Subramanian *et al.*, 2012b). Thus, to avoid harmful effects, screening of the mineral nutrient content of medicinal plants is highly essential. According to the World Health Organization (WHO, 1998), determination of these elements in medicinal plants is a part of quality control to establish their purity, safety and efficacy. In this regard, they recommend that medicinal plants, which form the raw materials for the finished products, must be checked for the presence of heavy metals and the level of mineral nutrients and further they regulate maximum permissible limits of these elements.

Many efforts have been made to analyze the mineral nutrient contents of medicinal plants from various parts of the world by applying several techniques, such as atomic absorption spectroscopy (Virgilio *et al*., 2012; Gupta *et al.*, 2014; Dghaim *et al.*, 2015), atomic emission spectrometry (Jia *et al*., 2011; Kwon *et al*., 2014), x-ray fluorescence (Desideri *et al.*, 2010), and inductively coupled plasma mass spectrometry (Tokalıoğlu, 2012; Bu *et al*., 2013). Even though many attempts have been reported for determination of mineral nutrient of medicinal plants from all over the world (Ajasa *et al*., 2007; Street *et al.*, 2007; Street *et al.*, 2008; Erna *et al.*, 2014; Rajan *et al.*, 2014); documented reports of medicinal plants growing in Malaysia are scarce (Ong and Norzalina, 1999). The present study was designed (1) to barcode four selected medicinal plants (*Murraya koenigii, Strobilanthes crispa, Justicia gendarussa* and *Centella asiatica*), commonly used in Malaysia and (2) to analyze six mineral nutrients (Ca, Fe, Mg, Mn, Zn, Cu) that could be found in the selected medicinal plants. Most of the traditional healers in Malaysia often prescribe mixtures of these medicinal plants in the raw form for diseases ranging from diarrhea; respiratory system and cancer; skin disease, malaria; high cholesterol level and high blood pressure (Duñg and Loi, 1991; Brinkhaus *et al.*, 2000; Sikder *et al.*, 2011; Gul *et al.*, 2012; Kadir *et al.*, 2014).

## 2. Material and Methods

### *2.1. Samples Collection*

Ten samples of medicinal plants were randomly collected from June to September 2013 from an eight-acre secondary forest located in Northern Peninsular of Malaysia (Table 1). Whole plant and/or parts of the plant were cut and collected with sharp scissors. The collected plant materials were then put in a sterile polyethylene bag and labeled before transported straight to the Molecular Ecology Laboratory, School of Biological Sciences, Universiti Sains Malaysia. Morphological identification of the plants collected was conducted with the help of local taxonomist from the School of Biological Sciences, Universiti Sains Malaysia and a book titled Photographic Atlas of Botany and Guide to Plant Identification authored by Castner (2005) (Table 1). The voucher samples were deposited in the herbarium of Universiti Sains Malaysia. Based on a search for the literature on the most common medicinal plants used by people in Peninsular Malaysia, only four plant samples were included for further analysis.

### *2.2. DNA Extraction, Amplification and Sequencing*

Approximately 200 mg fresh young leaves were ground to a fine paste and homogenized in a DNA extraction buffer [(50 mM Tris HCL pH 8.0, 25 mM EDTA pH 8.0, 150 mM NaCl, 40.0 ml H2O) and 1 g PVP 40 mw 40 000]. The mixture was made up to 100 ml with distilled water and the pH was adjusted to pH 5.0 with HCl. The ground material was then transferred to a 2 ml tube. The modification of the cetyl trimethylammonium bromide (CTAB) protocol by Cota- Sánchez (2006) was used for DNA isolation procedure of the leaf materials. Briefly, 500 µl of CTAB buffer was added into 2 ml tube contained ground material. The mixture of plant extract and CTAB buffer was then incubated overnight at 56°C and mixed intermittently by inversion. Subsequently, 250 µl of chloroform was added to the mixture and then mixed again by inversion. The tube was then spun at 12000 g for 5 minutes. Two layers of aqueous phase were formed and only the upper aqueous phase that contains

DNA was transferred to a new sterile tube. The DNA was precipitated with 50 µl 7.5M ammonium acetate (NaAc, pH 4.6) and two volumes of 95% EtOH before incubated at -20 0C for 1 hour.

The tube was centrifuged at 13000 rpm for 15 minutes to isolate the precipitates. The supernatant was removed and the DNA pellet was washed twice by adding 500 µl ice-cold 70% EtOH. After the tube was centrifuged for 10 minutes, the ethanol was poured off, and then the tube was centrifuged again for a few seconds before removing the remaining liquid using a pipette. The tube was then left to dry in the incubator (with the cap open) for 30 min, or until the remaining liquid had evaporated. Then, DNA pellet was then resuspended in Milli-Q water depended on the amount of isolated DNA. DNA was then incubated at 65°C for 20 minutes to destroy any DNases that may have been present and stored at -20°C. To obtain a high-quality DNA, free from polysaccharides and other metabolites that might interfere during PCR amplification, purified DNA concentration of each sample was estimated by ethidium bromide-stained band intensities against λ DNA.

Polymerase Chain Reaction (PCR) was used to amplify the target region of the gene in the nuclear or plastid genome of plants depending on the primer pairs used. The primers used in the present study are ITS2-2F, 5'-GCGATACTTGGTGTGAAT-'3, ITS2-R, 5'-GACGCTTCTCCAG ACTACAAT-3', psbA-F, 5'-GTTATGCATGAACGTAATGCTC-'3 (Sang *et al.*, 1997) and trnH-R, 5-'CGCGCATGGATTCACAAATC-'3 (Sang *et al.*, 1997). The PCR reaction consisted of 10X PCR reaction buffer, 25 mM MgCl2, 1.25 mM of each dNTPs, 1 unit of Taq polymerase, 10 µM of each primer, 20 ng genomic DNA and 30 µl Milli-Q water. Thermal cycling conditions (on a T100TM Thermal Cycler; BioRad, Singapore) were 35 x [94°C for 45 s, 40°C - 50°C (depends on the primer used) for 45 s, 72°C for 1 min] and a final incubation at 72°C for 10 min. Additional purification was performed on all samples using the Qiagen DNA Mini Kit (Germany) to remove contaminants, such as pigments, tannins and other polymerase chain reaction inhibitors). Purified PCR products were then sent to First Base Laboratories Sdn Bhd (1st BASE) for sequencing. At 1st BASE, DNA sequencing was performed using a BigDye® v3.1 Terminator (Applied Biosystems) sequencing kit, with approximately 20-30 ng of cleaned PCR products and 1.6 ρmol of primer (forward and reverse separately) in each reaction. Sequencing products were cleaned and then electrophoresed on an ABI 3100xl capillary sequencer following standard protocols.

### *2.3. Sequence Analysis and Species Identification*

The amplified partial of ITS2 and trnH-psbA region of collected medicinal plants was used in the basic local alignment search tool (BLAST) algorithm with the ITS2 and trnH-psbA gene of the same species using BLASTn from GenBank (available at http://blast.ncbi.nlm.nih.gov). In addition, a total of 83 (trnH-psbA) and 67 (ITS2) conspecific sequences of the collected plant samples were retrieved from GenBank. All sequences were collapsed in haplotypes using the Collapse software version 1.2 (Provan *et al.*, 2005). Haplotypes were then aligned using Clustal W version 2.0.12 (Larkin *et al.*, 2007) in combination with a total of 150 sequences of trnH-psbA and ITS2 retrieved from the GenBank database. All sequences were manually checked and trimmed in the Bioedit version 7.2.5 sequence editing program (Hall, 1999); alignments were then subsequently revised by eye in an effort to maximise positional homology. All positions containing gaps and missing data were eliminated from the data sets.

Species discrimination was evaluated according to tree-based analysis in which the Neighbour Joining (NJ) tree was adopted and performed in MEGA version 5.0 (Tamura *et al.*, 2011) with a K2P molecular evolutionary model and branch supports were determined using 10,000 bootstrap replicates. Successful identification using this method was inferred when sequences from the same species formed a monophyletic group.

The DNA barcoding gaps which defined as the spacer region between intra and inter specific genetic variations and identification efficiency was also implemented to investigate the breaks in the distribution of genetic pairwise distances and performed in Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.*, 2012). This method proposes a standard definition of the barcode gap and can be used to partition the data set into candidate species even when two distributions overlap (see Liu *et al.*, 2014). Sequences of trnH-psbA and ITS2 data set were uploaded to http: //www.abi.snv.jussieu.fr/public/abgd/abgdweb.html. For this analysis, we set up values for the prior P (prior maximum divergence of intraspecific diversity) ranging from 0.001 to 0.1 as if the P value is set too high, the whole data set will be considered as a single species (Puillandre *et al.*, 2012). The distance analysis was calculated based on a K2P analysis.

### *2.4. Sample Preparation for Analysis of Mineral Contents*

Successfully identified plant species (based on DNA characterization; see section above) were used in this analysis. Leaf samples were washed with fresh running water to remove dirt, dust and other contaminated agents and afterwards the leaf samples were re-washed with deionized water for more cleaning. They were dried in a shade at room temperature (22-25°C). The dried samples were crushed, powdered and homogenized using an agate mortar and pestle. The powdered samples were kept in polyethylene sampling bags separately until analyzed.

#### *2.4.1. Sample Digestion*

The microwave digestion of leaf samples was done in accordance with US EPA Method 3052. Dried and powdered of leaf sample of 0.5 g each was weighed directly into the digestion vessel liners. Nine mL of concentrated $HNO_3$, 0.5 mL of concentrated HF, 0.5 mL HCl and 1.0 mL of $H_2O_2$ were added to each vessel. The vessels were allowed to react for approximately one minute prior to sealing the vessel. Then, the vessels were sealed, placed in a rotor and heated in a microwave system for 20 minutes. The temperature profile was specified to permit specific reactions and incorporates reaching 190±5 ºC in approximately less than 5.5 minutes and remaining at 190±5 ºC for 9.5 minutes for the completion of reactions. After cooling, the vessel contents were filtered into a 100 mL volumetric flask and diluted with deionized water.

*2.4.2. Analytical Methods*

Mineral nutrients (Ca, Fe, Mg, Mn, Zn, and Cu) in the selected medicinal plant samples were analysed using Perkin Elmer Optima 7000 DV ICP-OES instrument equipped with WinLab32 for ICP Version 4.0 software. Sample introduction system was checked and absorption wavelength for the determination of each metal together with its linear working range was tuned. Three replications and sample sensitivity according to sample concentration were then selected. Sample sensitivity according to sample concentration was then selected. The concentration values were taken from the average of three reading. These samples can be directly introduced to the ICP-OES along with standards that were prepared earlier. The working standards used were 1, 2, 4 and 8 mg/L. The standards were prepared by serial volume/volume dilution in polypropylene vials. Standards preparation from stock solution was set based on the equation: $V_1 = M_2V_2 / M_1$, where, $V_1$, $V_2$, $M_1$ and $M_2$ represent: volume taken from stock solution (mL), volume of DI water (20 mL), concentration of stock solution (ppm) and concentration of working standard (ppm), respectively.

PerkinElmer NIST traceable calibration standards for ICP were used as the stock standards for preparing working standards. Certified reference standards for 18 elements in plant samples were used for validating the developed method. Multi-element standards (Merck, Germany) for ICP for 18 elements in 65% nitric acid (HNO3) were used as quality control check standards. As for digestion purpose, 49% HNO3, 37% hydrochloric acid (HCl), 30% hydrogen peroxide (H2O2) and 49% hydrofluoric acid (HF) were used.

## 3. Results and Discussion

*3.1. Sequencing Success*

A total of 158 sequences for two barcodes were analysed, from which 8 sequences (for both trnH-psbA and ITS2 genes) were successfully obtained from the collected medicinal plants and 150 sequences were obtained from GenBank. Each sample was successfully amplified by the 530 bp (ITS2) and 542 bp (trnH-psbA) fragments. In comparison, the amplification reactions were performed with full success (100%) for trnH-psbA, whereas ITS2 demonstrated lower reaction efficiency with successful amplification of 95% of samples. Several attempts per sample were needed for ITS2 adjustment of the PCR reagents (i.e., DNA template, dNTPs, Taq DNA Polymerase), which demonstrated significant variability in amplification success among different plant samples. High quality sequences were obtained for the amplified DNA samples in which trnH-psbA showed the highest efficiency (100% sequencing success) and ITS2 showed only 75% success.

The internal transcribed spacer of nuclear ribosomal DNA (nrDNA ITS) has been used as a universal barcode in discrimination of more than 6600 plant samples (Chen *et al.*, 2010; Liu *et al.*, 2012). In the present study, the ITS2 region showed lower sequencing success when compared to trnH-psbA region. Similar results were also reported in other studies in which the sequencing success was considered low (see e.g., Sass *et al.*, 2007; Hollingsworth, 2011; Tripathi *et al.*, 2013). Difficulty in amplifying and sequence has been identified as the main limitation for ITS (Hollingsworth *et al.*, 2011). This drawback thus has been used as an argument for considering ITS as less acceptable as a standard DNA barcode in some research (Bolson *et al.*, 2015).

*3.2. Performance of Markers in Species Discrimination*

BLASTn analysis for all sequences showed that of the four samples analysed, trnH-psbA was correctly identified 100% of the sequences both at species and genus level, respectively, indicating that the scientific names recovered from the BLASTn analysis matched the putative scientific names expected based on vernacular names and morphological identification (Table 1). Likewise, trnH-psbA gene consistently shows the highest success rate in species and genus identification (99.8%) when BLASTn analysis was employed for samples from wide range taxa. In contrast, the correct identification for ITS2 was lower both at the genus and species level which only 75% for the four collected samples and only 69.5% for samples retrieved from GenBank. Based on these results, the present study reveals that trnH-psbA is the most reliable DNA marker for the medicinal plants collected from Peninsular Malaysia (Table 1).

The variation of names used for the same medicinal plants throughout different races, ethnics and geographic range of the species is likely the most important reason for the mismatch in species identification by BLASTn and the corresponding plant species that based only on their vernacular names (Mankga *et al*., 2013). For example, in Malaysia, the name Pecah beling is mostly referred as *Baphicacanthus cusia*, *Strobilanthes crispus* or *Saricocalix crispus*. Other than Pecah Beling, the plant is also called Pecah kaca or Jin batu while in Indonesia it was called Pecah beling, Enyoh kilo, Kecibeling or Kejibeling. A study by Abu Bakar *et al.* (2006) showed that *Strobilanthes crispus* exhibit high antioxidant activity as well as anticancer properties by inhibiting the proliferation of cancer cell lines in vitro. Moreover, consumption of food products contained *S. crispus*, for example, tea could contribute to the additional antioxidant needed in the body to enhance defence system, as well as an additional nutraceutical supplement in patients diagnosed with breast cancer (Abu Bakar *et al*., 2006). However, inaccurate identification of plants will compromise the therapeutic value of medicinal plants, thus would endanger human health. In Iran, the case of *Bunium cylindricum* (adulterant) that was mixed with Zire-e-siah (*Bunium persicum*) and sold in the market has resulted in the degradation of the quality and efficacy of the drug (Joharchi and Amiri, 2012). Many cases of toxicity have been reported and mostly due to species misidentification (Viljoen, 2013). The situation, where a proliferation of herbal remedies that have been adulterated or substituted with other plant materials, has stressed the need for a quality control (Raterta, 2014).

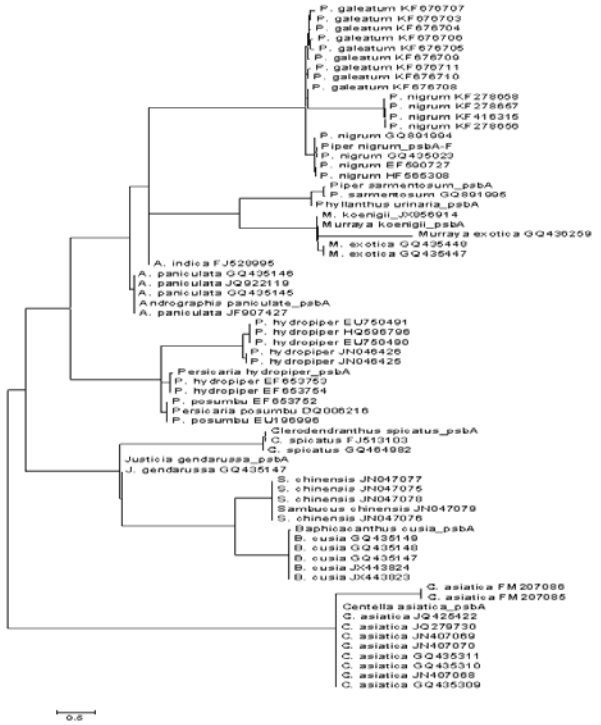
**Table 1**. List of medicinal plants analyses and BLAST analysis with the percentage of maximum identity for each sample

| No | Local name | DNA region | | | |
|---|---|---|---|---|---|
| | | ITS2 | | trnH-psbA | |
| | | Scientific name | Max Id (%) | Scientific name | Max Id (%) |
| 1 | Kari | *Murraya koenigii* | 99 | *Murraya koenigii* | 99 |
| 2 | Pecah Beling | *Sambucus chinensis* | 91 | *Baphicacanthus cusia* | 99 |
| 3 | Ganda Rusa | *Justicia gendarussa* | 99 | *Justicia gendarussa* | 97 |
| 4 | Pegaga | *Centella asiatica* | 98 | *Centella asiatica* | 99 |

### *3.3. Species Discrimination Based on Phylogenetic Trees*

An ideal DNA barcode must have adequate conserved regions for universal primer design, enough variability to be used for species identification (CBOL) and have a high ability to differentiate between closely related species, which will be only achieved when the genetic distance between species is significantly higher than within congeneric species (Hebert *et al.*, 2004; Mankga *et al.*, 2013). Barcoding studies normally use phylogenetic trees to assign species names and the most commonly utilized tree is neighbor joining, in which the assessment was based on phenetic distance and evolutionary information of a species (Liu *et al.*, 2014). Based on the neighbor-joining tree as shown in Figure 1, each barcode marker used was successfully separated each genus by representing a monophyletic clade, in which each clade appeared distinctly distant from other clades. For example, the clade containing genus Piper (*P. galeatum* and *P. nigrum*) and Murraya (*M. koenigii* and *M. exotica*) as displayed in *trnH-psbA* (Figure 1a) and ITS2 (Figure 1b) phylogenetic trees was well supported. The trnH-psbA genes demonstrated full successful identification (100%) both at genus and species level. However, the identification success of ITS2 barcode is only 98.7%. In this case, the positions of some species are ambiguous in which they were grouped within a different species and/or genus. For example, based on ITS2 gene, *Sambucus chinensis* (sequence obtained from this study) was largely separated from its origin where this species was located within the species *Baphicacanthus cusia* (sequence retrieved from GenBank) (Figure 1b).

Molecular systematic approaches have traditionally relied on comparing a limited number of the orthologous sequence to obtain estimates of species relationships across the tree of life (Edger *et al.*, 2014). Additionally, an assumption of phylogenetic analysis of nucleotide sequences is that each position is independent of other positions was always made in analysing phylogenetics relationship of a species (Alvarez and Wendel, 2003). The results of the present study demonstrated that the majority of sequence sites for ITS2 are not independently evolving, but rather are co-evolving with at least one other position in order to preserve the secondary structure of a molecule. Thus, in future studies, effort should be made to identify all co-evolving sites and appropriate adjustments are needed before employing ITS2 region as a phylogenetic marker.




(a)


(b)

**Figure 1.** Neighbour joining tree of selected medicinal plants collected from North Peninsular Malaysia and conspecific sequences retrieved from GenBank for (a) trnH-psbA *and* (b) ITS2. Sequences with red arrow show the species with ambiguous lineage.

### 3.4. Composition of Mineral Nutrients

The mean concentration levels of mineral nutrients in the four selected medicinal plants are summarized in Table 2.

#### 3.4.1. Calcium (Ca) and Magnesium (Mg)

The concentration level of calcium (Ca) is highest in *Strobilanthes crispa* with 5.76 mg/kg followed by *Centella asiatica* (5.71 mg/kg), *Justicia gendarussa* (5.26 mg/kg) and *Murraya koenigii* with 5.16 mg/kg (Table 2). High concentration of Ca is essential for all organisms due to its role in blood coagulation and necessary for proper functioning of the muscle and nervous systems (Straub, 2007). Meanwhile, the concentration levels of magnesium (Mg) in *M. koenigii*, *S. crispa*, *J. gendarussa* and *C. asiatica* were 1.37 mg/kg, 1.13 mg/kg, 1.72 mg/kg and 1.49 mg/kg, respectively.

Mg is a constituent of bones, teeth and enzyme cofactor in which it will be absorbed in the intestines and then transported through the blood to cells and tissues (Soetan *et al.*, 2010). The present study revealed that all plants analyses provide very low source of Mg as the necessary daily intake of Mg is 350 mg/day for men and 300 mg/day for women (WHO, 1998). Magnesium depletion will result in chronic or excessive vomiting and diarrhoea and acute magnesium deficiency result in vasodilatation, in which erythemia and hyperaemia will appeare a few days on the deficient diet (Champagne, 2008; Soetan *et al.*, 2010).

**Table 2.** Concentration levels of mineral nutrients (mg/kg) in the selected medicinal plants.

| | Plants | | | |
|---|---|---|---|---|
| Element (mg/kg) | Kari *Murraya koenigii* | Pecah Beling *Strobilanthes crispa* | Ganda Rusa *Justicia gendarussa* | Pegaga *Centella asiatica* |
| Ca | 5.16 ± 0.0 | 5.76 ± 0.0 | 5.26 ± 0.0 | 5.71 ± 0.0 |
| Cu | 0.01 ± 0.0 | 0.01 ± 0.0 | 0.01 ± 0.0 | 0.01 ± 0.0 |
| Fe | 0.15 ± 0.0 | 0.14 ± 0.0 | 0.15 ± 0.0 | 0.21 ± 0.0 |
| Mg | 1.37 ± 0.02 | 1.13 ± 0.0 | 1.72 ± 0.0 | 1.49 ± 0.02 |
| Mn | 0.0 ± 0.0 | 0.± 0. | 0.± 0.02 | 0.± 0. |
| Mo | 0.0 ± 0. | ND | 0.01 ± 0. | 0.01 ± 0. |
| Zn | 0.02 ± 0. | 0.03 ± 0. | 0.03 ± 0. | 0.01 ± 0. |

#### 3.4.2. Iron (Fe) and Manganese (Mn)

Among the selected medicinal plants, the mean concentration level of iron (Fe) varied between 0.14 and 0.21 mg/kg. Fe is important for the formation of haemoglobin and plays an essential role oxygen and electron transfer in a human body (Omokehide *et al.*, 2013). In cellular respiration, it functions as an important component of enzymes involved in biological oxidation (Soetan *et al.*, 2010). In the present study, the highest concentration level of Fe was found in *C. asiatica* (0.21 mg/kg) while *S. crispa* had the lowest concentration (0.14 mg/kg) (Table 2). WHO has recommended an intake of Fe of 10-20 mg/day for an adult and the permissible level of Fe in medicinal plants is 20 mg/kg (WHO, 1998). Even though WHO has formulated the guideline for quality assurance, the lack of knowledge and heavy use of medicinal plants may contribute to tissues damage and some diseases in humans. In contrast, deficiency of Fe has been reported to have a role in brain development and in the pathophysiology of restless leg's syndrome (Soetan *et al.*, 2010).

Manganese (Mn) concentration level can be found highest in *J. gendarussa* with mean concentration 0.37 mg/kg, while the lowest concentration was in *S. crispa* with mean concentration 0.04 mg/kg. *Murraya koenigii* and *C. asiatica* share the same concentration as presented in Table 2. Mn is an important element as a structural component of some enzymes as stated by (Saracoglu *et al.*, 2001). Mn is an essential trace element for the growth of plants and animals. Specifically, it is important in normal reproductive functions and normal functioning of the central nervous system (Devi and Sarma, 2013). Its deficiency produces severe skeletal and reproductive abnormalities in mammals. High concentration of Mn causes hazardous effects on lungs and brains of human (Son *et al.*, 2007). The recommended intake level for Mn is 11 mg/day for an adult and the maximum permissible limit of Mn in medicinal plants is 200 mg/kg (WHO, 1998).

#### 3.4.3. Zinc (Zn) and Copper (Cu)

*Justicia gendarussa* and *S. crispa* contained the highest zinc (Zn) concentration level of 0.03 mg/kg followed by *M. koenigii* (0.02 mg/kg) and *C. asiatica* (0.01 mg/kg) (Table 2). Zinc is essential to all organisms and has an important role in metabolism, growth, development and general well-being, as well as become a crucial co-factor for numerous enzymes in the body (Omokehide *et al.*, 2013). Zinc deficiency, particularly in children can lead to loss of appetite, growth retardation, weakness and even stagnation of sexual growth (Daur, 2015). Recommended limit of Zn in medicinal plants reported in WHO (1998) is 50 mg/kg, while its intake in food is 11 mg/day (Khan *et al.*, 2008). Thus, the concentration level of Zn observed in the present study was within the safe limit. While the concentration of Zn in the selected medicinal plants in the present study shows various ranges, the concentration of copper (Cu) was found in the same level in all four plants, which is 0.01 mg/kg (Table 2). Copper is an essential redox-active transition element that plays vital role in various metabolic processes and forms a component in many enzyme systems in the human body (Omokehide *et al.*, 2013). Due to the toxic properties of Cu, its quantity in plants should be very low. As reported by Negi et *al.* (2012), a high concentration of Cu causes metal fumes fever, hair and skin decolorations, dermatitis, respiratory tract diseases and some other fatal diseases in human beings. WHO permissible limit of Cu in medicinal plants is 10mg/kg, while its intake in food is 2-3mg/day (WHO, 1998).

## 4. Conclusion

Four medicinal plants from Peninsular Malaysia (*Murraya koenigii*, *Strobilanthes crispa*, *Justicia gendarussa* and *Centella asiatica*) were successfully identified based on DNA characterization at two target genes namely trnH-psbA and ITS2. Using the BLAST analysis and genetic distance method as inferred by phylogenetic tree, the present study suggests that

trnH-psbA is the best marker for identification of medicinal plants from Northern Peninsular Malaysia. The concentration levels of mineral nutrients (Ca, Fe, Mg, Mn, Zn, and Cu) in leaf samples of the selected medicinal plants were successfully determined using ICP-OES techniques. The observed concentration levels of the elements in the leaf samples of identified medicinal plants were found within the trace concentrations and significantly below the permissible limits. This indicates that the leaves of all medicinal plants in the present study are safe to use and consume. Further research on the use of other target regions for species discrimination and analysis of mineral concentration levels in other tissues of these medicinal plants would help to establish baseline data on these species.

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