ITS2 AND *trnH-psbA* IN IDENTIFYING *HIBISCUS* SPECIES, *MURRAYA PANICULATA* AND *MELALEUCA CITRINUS*

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THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
BACHELOR OF BIOTECHNOLOGY (HONOURS)

FACULTY OF HEALTH AND LIFE SCIENCE
INTI INTERNATIONAL UNIVERSITY
PUTRA NILAI, MALAYSIA

2018

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ACTKNOWLEDGEMENT

I finished this dissertation by couple of month. Time was spent in a through reading all literature review available on the issue.

I must extend my wholehearted gratitude to Dr. Geeta Selvarajah, my project supervisor. Dr Geeta gave me her valuable comment and share me her previous experience on doing this dissertation. My coursemates also helping me a lot in the lab session. While when I had problem on writing, they will give me some of their opinion. We are helping each other to complete this dissertation.

Last but not least, I want to bestow my thanks to my parent for their support. They gave me support when I was stress during the period I prepared this dissertationand unflinching moral support.

ABSTRACT

The identification of plant species is still relevant today. It is used in ecology, medical field and for conservation efforts. DNA barcoding is a method able to provide a standardized and efficient tool for identification. It involves specific DNA sequences such as ITS2 and *trnH-psbA* known as DNA barcodes which are compared with known barcode sequences found on databases. Previous experiments to obtain ITS2 from *Hibiscus* resulted in poor quality sequence probably because of the heterogeneity of the barcode. One of the goals of this study was to see if the same problem exists in other members of the same clade, using *Murraya panniculata, Hibiscus spp. and Callistemon citrinus* as samples. Additionally, the effectiveness of *trnH-psbA* was assessed in the identification of the Roside clade. This study also attempted to identify a hibiscus species which had morphological characteristics which could not clearly identify it at species level. DNA was extracted using CTAB buffer followed by amplification of the markers by PCR after genomic DNA bands have been obtained on an agarose gel. It was hard to grind the leaves of the *Hibiscus* since foaming occurred. The amplification of the markers also required several attempts and modifications of sample concentrations. Nonetheless enough PCR products were successfully obtained for sequencing, and good quality sequences were obtained for all the samples. However, traces showed some multiple peaks at specific locations in the ITS2 sequences of *Hibiscus rosa-sinensis* and *trnH-psbA* sequences of *M. paniculata*. High mismatch ratios between the forward and reverse sequence were also encountered for these sequences and a consensus sequence could not be obtained using DNA BASER software. In these cases, only sequences from unidirectional reads were used. Using BLAST, the identification of the *Hibiscus* species and *M. panniculata* were ambiguous unlike *C. citrinus*. Maximum likelihood tree and neighbor joining tree using both markers could differentiate each species. Like the *H. rosa-sinensis and M. panniculate*, the unknown *Hibiscus* may be a hybrid; as the nuclear locus identified it as *Hibiscus fragilis* and the chloroplast loci identified it as *Hibiscus syriacus*.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
μL	Microlitre
μM	Micromolar
ug/mL	Microgram per microlitre
%	Percentage
BLAST	Basic Local Alignment Search Tool
COI	*Cytochrome c oxidase 1* gene
CTAB	Cetrimonium bromide
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
dNTP	Deoxynucleotide
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetraacetic Acid
g	Gram
HCl	Hydrochloric acid
hr	hour
ITS	Internal transcribed spacer
ITS2	Internal transcribed spacer 2
kb	kilobases
M	Moles
matK	Megakaryocyte-Associated Tyrosine Kinase
MEGA 7.0	Molecular Evolutionary Genetics Analysis

$MgCl_2$	Magnesium Chloride
mg/mL	Miligram per millilitre
min	Minute
mL	Millilitre
mM	Millimolar
MW	Molecular weight
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
nmoles	nanomoles
PCR	Polymerase chain reaction
pH	Potential of hydrogen
rbcl	Ribulose bisphosphate carboxylase
RNA	Ribonucleic acid
rpm	Revolutions per minute
sec	Second
TBE buffer	Tris/Borate/EDTA buffer
TE buffer	Tris/Borate buffer
TER	Tris/EDTA buffer with RNase A
w/w	Volume of solute in millilitres per 100 millilitres
w/v	Weight in grams of a solute per 100 millilitres

CHAPTER 1

INTRODUCTION

Approximately 1.7 million species have been identified through the conventional taxonomic method that is using morphological traits (Mohamad et al., 2014). However, this figure is not totally reliable because this method is very subjective, thus many problems were encountered and it was a slow process (Mohamad et al., 2014). Accurate and quick identification of species is extremely crucial in scientific fields such as ecology, epidemiology, conservation, evolution (Kress, García-Robledo, Uriarte & Erickson, 2015) and recently even in crime detection for example illegal trading of timber (Hartvig, Czaka, Kjaer, Nelson & Theilade., 2015). Thus, DNA barcoding is used.

Several DNA barcodes have been proposed for land plants namely *trnH-psbA, matK, rbcL, rpiCl, ycf5,* ITS and ITS2 (Hui et al., 2010). The *matK* and *rbcL* combination is considered as a core two marker barcode because *rbcL* can be easily recovered and *matK* has good discriminatory power (Hollingsworth, Graham, & Little, 2011). However, this combination has drawbacks that makes its utility in identification questionable (Hollingsworth et al., 2011). *matK* does not always amplify with the current available primers and *rbcL* lacks sequence variation (Hollingsworth et al., 2011). Thus, DNA barcodes which exhibit higher levels of sequence variation and can be easily amplified may improve the ability to identify species. The barcodes having these characteristics are nuclear ITS and *trnH-psbA* (Hollingsworth et al., 2011; Huyen, Thi, Truong, Duy, & Hoang, 2017).

The success of using this technology, DNA barcoding, also relies on sequence homogeneity of barcode which ITS2 may not have. Being a nuclear gene it may possess bi-parental inheritance, meaning two nuclear ribosomal ITS2 sequences that differ exist together in an organism if it is a heterozygote (Hughes, Morris, & Reboredo-Segovia, 2015). Heterogeneity posed difficulties for Sanger sequencing as double or multiple nucleotide peaks were present in the sequence traces (Woo *et al.,* 2010).

The problem of poor sequence quality of ITS2 has been reported in Hibiscus, and it is suspected that it is due to the existence of heterogenous sequences since it is known that *Hibiscus rosa-sinensis* may have undergone hybridization with other plant species and thus result in many variations among the *Hibiscus* plant species (Singh & Khoshoo, 1970). Whether this is a problem that exists in other members of the same clade, rosids, is also a question.

Thus the aims of this study were to:

- Determine whether good quality sequences of ITS2 can be obtained from other plants of the same clade as Hibiscus, i.e., rosids, which are *Muraya paniculata* and *Melaleuca citrinus.*
- Determine whether ITS2 and *trnH-psbA* can effectively differentiate between *Hibiscus spp., Muraya paniculata* and *Melaleuca citrinus*
- Identify an unknown *Hibiscus* species using ITS2 and *trnH-psbA*

CHAPTER 2

LITERATURE REVIEW

2.1 *Hisbiscus rosa-sinensis*

The known *Hibiscus* species in this study is *Hibiscus rosa-sinensis.* It is a widely distributed plant which has many variants and used as a garden ornamental plant worldwide (Ross, 2003). It belongs to the family Malvaceae and it is believed to have originated from the East Asia, mostly China (Lim 2014).

H. rosa-sinensis is a shrub which can grow up to 20 feet (Ross, 2003). It is multi-branched plant where the branches are long and thin and spirally arranged along the stem (Ross, 2003). The *H. rosa-sinensis*'s flowers are large, conspicuous and trumpet shaped single ones which can reach 18 cm breadth and located in the axils of the upper leaves (Ross, 2003). The corolla of the flower can be of different colours such as rosy red, white, reddish, purplish and orange-yellow (Lim, 2014). This plant normally remains upright and always retains its green leaves (evergreen) (Lim, 2014). The leaves are dark green in color and ovate with a serrated leaf margin (Lim, 2014).

Besides being used as an ornamental plant (Ross 2003), the plant possesses medical properties such as anti-depressant (Pallavi, Rupali & Yogesh, 2012), antibacterial (Ruban, & Gajalakshmi, 2012), cardio-protective (Gauthaman, et al., 2006), wound healing (Bhaskar, & Nithya, 2012), anti-dyslipidaemia and anti-oxidant activities (Kumar, *et a.l,* 2013). It can also be used to stimulate menstrual flow (Burkil, Birwistle, Foxworthy, Scrivenore, & Watson, 1935). It treats gonorrhoea (Whistler, 1985), diarrhoea and promotes childbirth as well as milk production in the mammary gland (Kobayashi, 1976).