DNA BARCODING IN *Hibiscus rosa-sinenensis* VARIANTS USING *matK* AND *trnH-psbA* BARCODES

MARCELLA MEIA ANAK GARRY ENCHANGAN

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MARCELLA MEIA ANAK GARRY	DR GEETA SELVARAJAH
I14004775	(SUPERVISOR)

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ABSTRACT

Hibiscus rosa-sinensis is a versatile plant species of many uses ranging from medicinal to a food source and even industrial. There are multiple variants of *H. rosa-sinensis* which confers different levels of antimicrobial, antioxidant and wound healing activity as each variant consists of different levels of phytochemicals. Thus, accurate identification is critical so that each of their medicinal properties can be utilized to the fullest degree. However, the variants only differ in flower colours and shapes but are similar looking shrubs. The flowers of the *H. rosa-sinensis* are not present all the time and are short lived, leading to the difficulty in accurately identifying each variant based solely on morphology. Thus, DNA barcoding is a possible complement to traditional morphology based identification and discrimination. In this study, the combination of *trnH-psbA* and *matK* sequences were used as barcodes to discriminate three different variants of *H. rosa-sinensis*. The variants that were sequenced under this study are provisionally identified as *H. rosa-sinensis* Fijian white, *H. rosa-sinensis* with a single layer of pink petals and *H. rosa-sinensis* with a single layer of orange petals. DNA extraction using a modified Edward's method, amplification and sequencing were successful, with good quality sequences being generated in most cases. The probability that these sequences were not pseudogenes or contaminants were verified using BLAST and DOGMA. The phylogenetic tree generated showed that the *trnH-psbA* marker has higher discriminatory power than the *matK* marker due to its ability to assess intraspecific relationship of the variants with high confidence. As there are few studies that had been done on DNA barcoding of *H. rosa-sinensis* variants, the DNA sequences of this current research will contribute to the public DNA database and hopefully aid in the future identification and differentiation of *H. rosa-sinensis* variants.

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AIC	Akaike information criterion
BLAST	Basic Local Alignment Search Tool
COBL	Consortium for the Barcode of Life
DNA	Deoxyribonucleic acid
H. rosa-sinensis	*Hibiscus rosa-sinensis*
H. shizopetallus	*Hibiscus shizopetallus*
MEGA 7	Molecular Evolutionary Genetic Analysis
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
RAPD	Random Amplifies Polymorphic DNA
rbcl	RuBisCo large subunit
RNA	Ribonucleic acid
RNAse	Ribonuclease
SDS	Sodium dodecyl sulfate
TBE buffer	Tris/Borate/EDTA buffer
TE buffer	Tris/EDTA buffer
TER	Tris/EDTA buffer RNase A

CHAPTER 1

INTRODUCTION

Recently, there is a global increase in the usage of plant-based medicine which is a large source of income in countries such as China and Brazil as reported by World Health Organization (WHO) cited in Marcial-Quino, Mendoza-Espinoza & Sierra-Palacios, 2015. Malaysia can exploit this opportunity as we have an abundance of medicinal plants including *Hibiscus* which can be of used in the pharmaceutical industry.

Hibiscus is a genus of flowering plant from the family of Malvaceae, consisting of about 275 species in the tropics and subtropics regions alone (Prasad 2014). This genus of plant has multiple uses, from medicinal to industrial uses (Awang, Hamidi, Mohammed & Muhammad, 2013; Mulchand & Rajendra, 2015; Sumanta, Debjit, Nuni & Seru, 2016). The species *Hibiscus rosa-sinensis* specifically, are known for their medicinal properties such as antimicrobial activity, antifertility activity, antioxidant activity, antitumor activity and even wound healing (Goldberg et al., 2015; Kandhare et al., 2012; Raduan et al., 2013; Reena, Aditi, Dharmesh & Anju, 2012; Khudhr, Sajet, Basim & Abd-Alkhalik, 2015; Sumanta et al., 2016). This is due to their phytochemicals such as flavonoids and vitamins found in varies parts of the plant (Salem, Olivares-Pérez & Salem, 2014; Shashi, Rachna, Alka & Rashmi, 2016). Reena et al. (2012), showed that different variants of *H. rosa-sinensis* have different levels of antimicrobial activity towards different species of pathogens which is due to the variations in secondary metabolites of each variants of *H. rosa-sinensis*.

H. rosa-sinensis varieties have flowers ranging in colours from white or yellow to pink or red with double or single petals (Prasad, 2014). When not in bloom, these assortments of *H. rosa sinensis* are difficult to differentiate based solely on their morphological features. As these variants of *H. rosa-sinensis* have different phytochemicals in their flowers, leaves, stems and roots, they show different antioxidant and antibacterial activity making accurate identification critical for pharmaceutical application (Reena, Aditi, Sachin & Anju, 2015; Sumanta et al., 2016).

To complement the traditional identification via morphological features, DNA barcoding can be utilized. This is an identification technique using short DNA sequences for a more accurate identification of organisms (Bruni et al., 2012). However, up until 23rd of January 2017, there were only 6 *H. rosa-sinensis* DNA sequences with the *matK* and *trnH-psbA* loci on GenBank, with no specification on the morphology of the *H. rosa-sinensis* used. The DNA sequences obtained from the barcoding in this experiment is contributed to the public DNA database and will hopefully aid in the identification and differentiation of *H. rosa-sinensis* variants. In this study, DNA barcoding of three *H. rosa-sinensis* variants will be carried out using the loci *matK* and *trnH-psbA*. The three morphological variants are *H. rosa-sinensis* Fijian, *H. rosa-sinensis* with a single layer of pink petals and *H. rosa-sinensis* with one a single layer of orange petals. The aims of this study is to determine DNA sequences of *matK* and *trnH-psbA* for the three *H. rosa-sinensis* variants and to determine whether the markers used are able to differentiate the variants.

CHAPTER 2

LITERATURE REVIEW

2.1 *Hibiscus rosa-sinensis*

Known as Chinese hibiscus or tropical hibiscus, *H. rosa-sinensis* is from the large plant family of Malvaceae and the genus *Hibiscus* (Gilman, 1999). *H. rosa-sinensis* is a widely planted shrub with glossy dark green leaves and comes in various varieties, cultivars and hybrids (Gilman, 1999; Prasad, 2014; Reena et al., 2015). Their structures which can be used stretch from their buds to their leaves and even their roots (Nayak, Ashe, Rauta, & Nayak, 2015; Nirmaladevi, Kalpana, Kavitha & Padma, 2012).

H. rosa-sinensis contains numerous active compounds called phytochemicals such as alkaloids, polyphenolics, tannins, steroids and essential oils which are responsible for their medicinal properties (Reena et al., 2015; Salem, Olivares-Pérez & Salem, 2014; Shashi & Rachna, 2014). Reena et al. (2012) found that their leaves, roots and stems have a substantial amount of flavonoids which corresponds to the antioxidant activities and the flower have soothing properties which are used in easing menstrual cramps. Extracts of *H. rosa-sinensis* was found to have antitumor activity, antifertility activity, wound healing and antidiabetic activity (Jana, Das, Ray, Mandal, Giri, & Bhattacharya, 2013; Reena et al., 2012; Salem et al., 2014). Moreover, flower extracts of *H. rosa sinensis* was also found to interfere with the formation of kidney stone (Nirmaladevi et al., 2012). Nayak et al. (2015) uncovered an environmentally-friendly route of extracting silver nanoparticles from the petals of *H. rosa-sinensis* which acts as reducing, capping and stabilizing agent for biomedical applications. These are only a few reasons why *H. rosa-sinensis* is a plant of high value.

Variants of *H. rosa-sinensis* have different phytochemical contents and levels and thus have different medicinal activities (Falguni & Subrata 2012; Khan et al., 2014; Reena et al., 2015; Salem, Olivares-Pérez & Salem, 2014). Compared to the other variants of *H. rosa-sinensis*, the red variants had the highest antioxidant capacity (Khan et al., 2014). In an evaluation by Raduan et al. (2013) the *H. rosa sinensis* white variant was shown to be more potent in anti-inflammatory action compared to the red variant of *H. rosa sinensis* due to their different phytochemical content in different parts of the plants.

2.2 *Hibiscus rosa-sinensis* VARIANTS RELATIONSHIP

According to the International Hibiscus Society website (http://internationalhibiscussociety.org/new/), *H. rosa-sinensis* has over 9000 cultivars that had been reported. Various molecular identification techniques have been used to identify and determine the relationship of various *H. rosa-sinensis* variants. Prasad (2014) used the random amplifies polymorphic DNA technique and found that there was a significant variation within the flower varieties.

The variations may be due to environmental influence or species hybridization. Most of *H. rosa-sinensis* variants are a result of hybridization with other *Hibiscus* species such as *Hibiscus kokio, Hibiscus denisoni* or *Hibiscus genevii* which are all sexually compatible with *H. rosa-sinensis* (Braglia et al. 2010).

Braglia et al. (2010) utilized the amplified fragment length polymorphism technique to elucidate the genetic relation of *H. rosa-sinensis* cultivars and also its relation with other Hibiscus species. The variants of *H. rosa-sinensis* were grouped into ancients, wild and commercial with the commercial cultivars having the highest number of variant as a result of artificial selection.

2.3 DNA BARCODING

Traditional identification of plants uses morphological approaches to identify a whole plant. However, even taxonomic experts encounter with specimens that cannot be identified reliably. Hence, accurate, subjective and swift identification practice such as DNA barcoding is employed. DNA barcoding is a technique introduced by Paul Herbert from the University of Guelph in 2003 to effectively and rapidly identify species by using highly variable, short standardized DNA markers (Galimberti et al., 2013).

Identification by DNA barcoding has many uses such as authentication of plant products, food safety, quality control and recognizing invasive species. García-Robledo, Erickson, Staines, Erwin and Kress (2013) used DNA barcoding to study the species interaction between insects and plants.

DNA barcoding was first utilized to identify animals by using DNA sequence in a region of the mitochondrial *cytochrome c oxidase I* gene in addition to traditional morphology-based identification (Saarela, Sokoloff, Gillespie, Consaul & Bull, 2013; Sun et al., 2012; Tripathi et al., 2013; Velzen, Weitschek, Felici & Bakker, 2012). Nonetheless, this DNA sequence cannot be applied in barcoding of plants due to the low substitution rate of plant mitochondria (Saarela et al. 2013; Tripathi et al. 2013; Yang et al. 2012). Therefore, the chloroplast genome is employed for barcoding of plant species as it is haploid and generally uniparentally inherited (Dong et al., 2012; Vijayan & Tsou, 2011). Chloroplast genome also eases PCR amplification and sequencing which further simplifies the barcoding process (Vere et al., 2012; Dong et al., 2012; Tripathi et al., 2013; Urumarudappa et al., 2016). An ideal barcode must have universality, high sequence quality and high discriminatory power (Hollingsworth, Graham & Little, 2011). Plant nuclear genes cannot be used in DNA barcoding as they are highly variable and occur in multiple copies, resulting in difficulty of designing universal primers (Vijayan & Tsou, 2011).