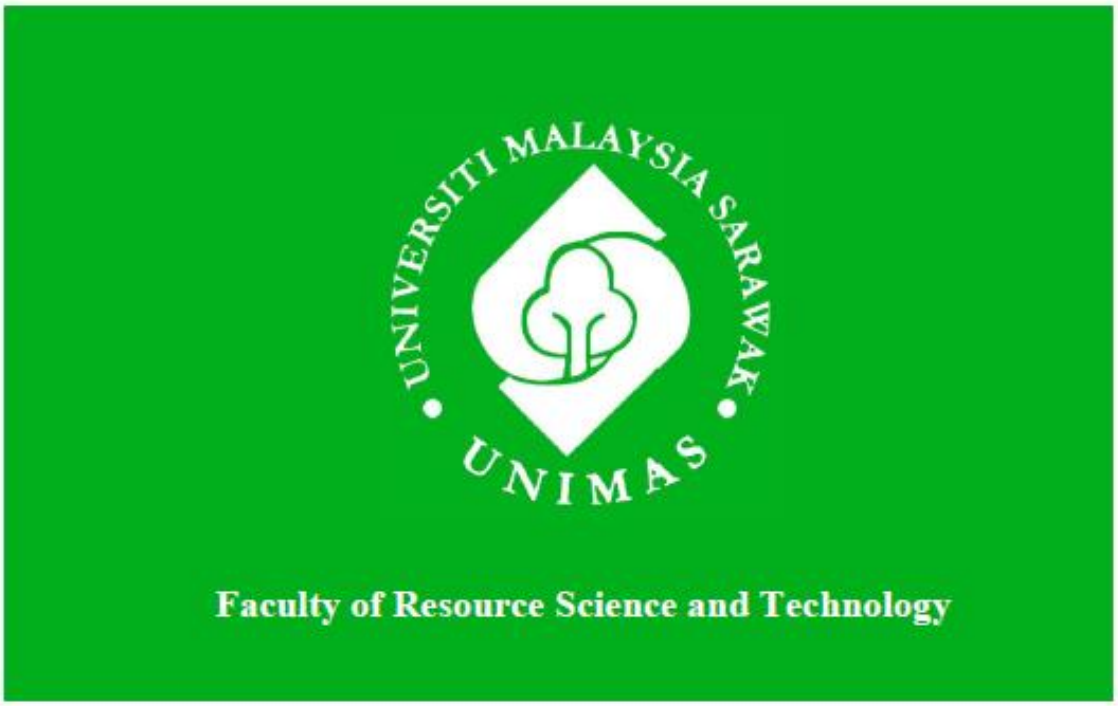

The Use of Internal Transcribed Spacer (ITS) as Marker for Rapid Identification of *Dolichandrone spathacea*

Cheah Men How

35692

Bachelor of Science with Honours
(Aquatic Science and Resource Management)
2015

UNIVERSITI MALAYSIA SARAWAK

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The Use of Internal Transcribed Spacer (ITS) as Marker for Rapid Identification of *Dolichandrone spathacea*

CHEAH MEN HOW

This project is submitted in partial fulfilment of the requirement for the degree of Bachelor of Science with Honours (Aquatic Resource Science and Management)

Faculty of Resource Science and Technology

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2015

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Declaration

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning.

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List of Abbreviations

bp	Base pair
CTAB	Cetyltrimethyl Ammonium Bromide
DNA	Deoxyribonucleic acid
dNTP mix	Deoxyribonucleic Triphosphate Mix
FRST	Faculty of Resource Science and Technology
ITS	Internal Transcribed Spacer
mM	Millimolar
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
TBE	Tris-borate-EDTA
µL	Micro liter

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ABSTRACT

The identification of mangroves is usually depends on the external morphological characteristics such as leaf shape, flower color and fruit morphology. However, this method is tedious and difficult especially when some characteristics that only appear in certain season. Molecular procedure such as DNA barcoding emerged as a powerful tool to solve species problem. DNA barcoding is a molecular procedure that uses a short length of DNA sequence as a marker to identify species in question. This study is designed to sequence the internal transcribed spacer (ITS) region of nuclear ribosomal DNA from the leaf samples of mangrove associate tree which obtained from estuary around External Laboratory, UNIMAS at Kota Samarahan and to use ITS region as a marker for rapid species identification. Approximately between 600bp and 700bp ITS region had been sequenced from leaf samples and it matched 99% to *Dolichandrone spathacea* (Accession: KJ161167). Thus, it is confirmed that the tree species is *D. spathacea*. Both Neighbour-Joining (NJ) and Maximum Parsimony (MP) phylogenetic trees showed that *D. spathacea* is monophyletic, whereas other true mangrove species formed a separate clade. This study showed that ITS region is suitable to be used for rapid identification of *D. spathacea*.

Key word: DNA barcoding, ITS region, mangrove associate tree, identification, *Dolichandrone spathacea*.

ABSTRAK

Identifikasi pokok bakau biasanya tergantung pada ciri-ciri morphologi luaran seperti bentuk daun, warna bunga, dan morphologi buah. Tetapi, cara ini lumit dan susah apabila ciri-ciri tertentu hanya muncul mengikut musim. Prosedur molecular seperti DNA barkoding merupakan prosedur berguna dalam penyelesaian masalah identifikasi spesies. DNA barkoding merupakan satu prosedur molekular yang menggunakan jujukan DNA pendek sebagai penanda untuk kerja identifikasi spesies dalam kajian. Kajian ini direka untuk mendapatkan bahagian Internal transcribed spacer (ITS) dalam nuclear ribosomal DNA daripada sampel daun pokok bakau ikutan yang berasal dari External Laboratory, UNIMAS di Kota Samarahan dan menggunakan bahagian ITS sebagai penanda untuk identifikasi spesies secara cepat. Kira-kira antara 600bp dan 700bp bahagian ITS telah didapatkan dari sampel daun dan ia 99% padan dengan Dolichandrone spathacea (Accession: KJ161167). Dengan ini, pokok bakau ikutan tersebut disahkan adalah D. spathacea. Pokok filogenetik Neighbour-Joining (NJ) dan Maximum Parsimony (MP) menunjukkan bahawa D. spathacea adalah monofiletik dan spesies pokok bakau sejati yang lain menjadi satu klad berlainan. Kajian ini menunjukkan bahawa bahagian ITS sesuai untuk digunakan untuk kerja identifikasi D. spathacea secara cepat.

Kata kunci: DNA barkoding, bahagian ITS, pokok bakau ikutan, identifikasi, Dolichandrone spathacea.

2.0 Introduction

Mangrove forest is an important ecosystem that usually located in estuaries of the tropical regions the world. Mangrove plants can be divided into true mangrove species and mangrove associated species. In Malaysia, true mangrove species comprises *Avicennia*, *Sonneratia*, and *Bruguiera* can be found in estuaries. In addition, mangrove associated species from genera such as *Euphorbiaceae*, *Fabacea* and *Arecaceae* can also be found within certain parts of the estuaries (Kasawani *et al.*, 2007; Wah *et al.*, 2011). Khairul (2000) reported that the pioneer mangrove species such as *Avicennia* and *Sonneratia* usually can be found at seaward border. For landward direction, these mangrove species are then replaced by *Bruguiera* and *Rhizophora* species (Khairul, 2000).

Mangrove ecosystem is a very important asset as it gives numerous ecological services and benefits to all life. It supports many different species of organisms, including birds, invertebrates, mammals, and fishes either from marine or freshwater environment (Alongi, 2009; Karleskint *et al.*, 2013). Besides, mangrove ecosystem also helps to reduce coastal erosion and flooding, as well as to provide and regenerate nutrients necessary for both terrestrial and aquatic organisms (Kasawani *et al.*, 2007). Economically, mangrove is important as timbers which harvested for firewood, charcoal and various construction works (Mastaller, 1997; Rusila Noor *et al.*, 2006; Kasawani *et al.*, 2007).

Dolichandrone spathacea is a mangrove associate species which normally found in estuaries. Although it is not a true mangrove species, *D. spathacea* was commonly found inhabit in estuaries and capable of adapting the extreme environmental conditions in estuaries. *D. spathacea* is widespread and was listed as the Least Concern species in IUCN Red List

(Duke *et al.*, 2010). Although the range of distribution of *D. spathacea* is extensive and it can be found in many countries, it is appeared to be rare in some parts of its range (Duke *et al.*, 2010). For instance, this species is rare in Vietnam; only found in certain parts in Australia and not abundant as compared to other countries (Duke *et al.*, 2010).

Mangrove areas along the river near UNIMAS External Laboratory support a great variety of plant species. Mangrove plant taxonomy depends on several key characters, for examples, leaf shape, arrangement of leaves, the shape and color of flowers, the shape and color of fruits, etc. However in certain occasions, especially during non-flowering and fruit seasons, it is difficult to identify a mangrove species as key characters are missing. Therefore, genetics becomes an important tool in species identification works. The objective of this study is to determine whether ITS region could be useful for rapid identification of *D. spathacea*.

2.0 Literature Review

2.1 Importance of Mangrove Ecosystem

Mangrove ecosystem is as important as much as terrestrial and marine ecosystems. The most essential component in mangrove ecosystem is the mangrove plants. Mangroves are heterogeneous group of intertidal plants that found at sheltered coastal regions as well as inner river systems where tidal influence exists (Saenger, 2002; Sahu & Kathiresan, 2012). Mangroves included two categories, namely: true mangroves and mangrove associates (Wang *et al.*, 2010). Wang *et al.* (2010) suggested that these two categories can be distinguished by determining the differences in terms of leaf traits and osmotic properties. Mangroves grow in estuaries where fresh water and sea water meet (Hogarth, 2007). Mangrove plants are well adapted to the constant changing environmental factors in estuaries, such as water salinity and dissolved oxygen fluctuations, changing pH level, tidal effects, water clogging, hot weather as well as unstable muddy sediments (Hogarth, 2007).

Hogarth (2007) stated that mangroves are a source of primary production as the mangroves provide nutrients in the form of leaf litter, reproductive products, twigs, and even dead trees. Therefore, mangroves are fuelling virtually the entire ecosystem. Mangrove ecosystem also functions as a breeding ground for many fish species and a shelter for their juveniles to avoid from predation (Karleskint *et al.*, 2013). Next, mangrove trees are capable of trapping sediment brought in by river and tide, and this will help to consolidate the mud in which they grow (Horgarth, 2007). Indirectly, this also provides a substrate on which mollusks such as oysters and barnacles can settle on, a habitat for insects, and nesting sites for many bird species (Horgarth, 2007).

Mangrove ecosystem provides many ecological services that are beneficial to the survival and well-being of living beings, which can be categorized into four aspects. There are supporting, provisioning, regulating, and cultural services (Walters *et al.*, 2008). Firstly, supporting services provided by mangrove ecosystem included soil formation, photosynthesis, primary production, water cycling and nutrient cycling while provisioning services are referred to the natural products that were produced by mangroves (Walters *et al.*, 2008). Next, regulating services include climate regulation, water quality maintenance, and the biodiversity maintenance for ecosystem function and resilience (Walters *et al.*, 2008). Moreover, Mangrove ecosystem acts as a natural barrier which helps to coastal communities from storm, cyclone, tsunami, flooding, and coastal erosion (Kasawani *et al.*, 2007; Kerr & Baird, 2007; Walters *et al.*, 2008).

Besides, mangrove ecosystem is also economically important and served as a source of income for indigenous people too. For example, mangroves are harvested by human in order to gain timber for construction, charcoal, firewood, fishing poles, pulp and tannin (Mastaller, 1997; Ashton & Macintosh, 2002). Rusila Noor *et al*. (2006) also pointed out that mangrove ecosystem is important for fishery activities, for example, high valued species such as *Lates calcifer*, *Scylla serrata*, and *Polynemus sheridani* are all depend on mangrove ecosystem. Furthermore, certain parts of mangroves also can be used as food, for instance, the pickled fruit of *Avicennia officinalis* can be eaten and parts of *D. spathacea* can be used as medicine (Rusila Noor *et al.*, 2006; Choudhury *et al*., 2011).

2.2 Origin and Evolution of Mangroves

The evolutionary history of mangroves is yet to be fully understood. Based on the fossil evidence, Mastaller (1997) reported that mangrove species are not evolved from ancestral specialized branch salt-tolerant fern species but evolved from flowering plants in freshwater swamps. According to Sahu and Kathiresan (2012), the first appearance of mangroves was believed to be as early as 80 million years ago, possibly just after the appearance of angiosperms. It is also believed *Avicennia* and *Rhizophora* were possibly the first genera to evolve, appearing near the end of the Cretaceous period (Sahu & Kathiresan, 2012). Sahu and Kathiresan (2012) also stated that the origin and spread of mangroves are not fully understood yet, whether mangroves are originated from the Malaysian peninsular and then spread to a region between Australia and Papua New Guinea, and or between Malaysia and Northern Australia.

2.3 Classification of *Dolichandrone spathacea*

Dolichandrone spathacea is a mangrove associate or 'semi-mangrove' plant that commonly found in mangrove ecosystem. Santisuk (1983) stated that the leaves of *D. spathacea* are oppositely arranged, imparipinnate, and the leaflets are slightly oblique at the base. Santisuk (1983) described *D. spathacea* as trees without pneumatophores, large white flowers, and capsule linear compressed, with many thick, corky, lateral-winged seeds. *D. spathacea* leaves was showed in Figure 2.1. As reported by Duke *et al*. (2010), *D. spathacea* lived at the upstream part of estuaries where the river systems are influenced by tidal changes. This mangrove species can be found in most South East Asia countries, such as Thailand, Cambodia, Vietnam, Brunei Darussalam, Malaysia, Indonesia, Singapore, and Philippines (Duke *et al.*, 2010). It also can be found in India, Sri Lanka, northeast tip of Australia and Papua New Guinea, Solomon Islands and to New Caledonia, and Palau (Duke *et al.*, 2010). Besides that, Duke *et al.* (2010) also mentioned that *D. spathacea* is a small, extensively-branched, rapid growing tree, which often assiociated with true mangrove species such as *Acanthus ilicifolius* and non-mangrove *Nypa fruticans*.

D. spathacea is a member of Phylum Tracheophyta, Class Magnoliopsida, Order Scrophulariales in Kingdom Plantae (Duke *et al.*, 2010). *D. spathacea* is commonly known as 'mangrove trumpet tree', 'Tui' in Malay language, 'Diya danga' in Sinhalese, 'Vilpadri' in Tamil, 'Khe Pa' in Thai and 'Nan Ya Mao Wei Mu' for Chinese. Like any other mangrove species, the wood of *D. spathacea* can be used to make match-sticks, toys, and charcoals. Next, Choudhury *et al.* (2011) stated that *D. spathacea* possessed some medicinal value. For example, in Indonesia, the leaves of *D. spathacea* are traditionally used to treat thrush; while in Philippines, the parts of this species can be used as traditional ingredient to treat nervous

diseases and flatulence (Choudhury *et al.*, 2011). The classification of *D. spathacea* is shown as in Figure 2.2.

D. spathacea is listed by IUCN Red List as the Least Concern species with their distribution is widespread and abundant (Duke *et al*., 2010). However, the population size of *D. spathacea* is decreasing and there is about 23% decline of *D. spathacea* in mangrove areas worldwide within this species range since 1980 (Duke *et al*., 2010). There are several causes contributed to this phenomenon. According to Duke *et al.* (2010), one of the major threats is the rise of sea level. As the sea level rises, the habitat requirements of every species will be disturbed and at the same time, each species will suffer high mortality rate at their present locations and forced to re-establish themselves at higher elevations in areas that were landward zones formerly (Duke *et al*., 2010). Next, the decreasing trend of *D. spathacea* population is also caused by habitat destruction and rapid unsustainable development of mangrove areas.

There are many reasons for mangrove area removal, which included clearing for shrimp farms, agriculture, fish ponds, rice production and salt pans, as well as for the purpose of urbanization and industrial development (Duke *et al*., 2010). Projects such as construction of roads, coconut plantations, ports, airports, and recreational places are also contributed to the loss of mangrove areas (Duke *et al*., 2010). Pollution from sewage effluents, solid wastes, siltation, leaked oil, agricultural wastes, and urban runoff contribute to the deterioration of the mangrove areas too (Duke *et al*., 2010). Moreover, climate change is also considered as a key factor, especially at the edges of distribution range of the species (Duke *et al*., 2010). Another reason for the population drop is natural causes. Natural disasters such as cyclones, hurricane and tsunamis are capable of destroying large patch of mangrove areas (Duke *et al*., 2010).

Figure 2.1: Leaves of *D. spathacea* (adapted from http://amap-collaboratif.cirad.fr/pages_logiciels/Mangrove_web/especes/d/dolsp/dolsp_03.jpg)

Kingdom Plantae

Phylum Tracheophyta

Class Magnoliopsida

Order Scrophulariales

Family Bignoniaceae

Genus *Dolichandrone*

Species *Dolichandrone spathacea*

Figure 2.2: Classification of *D. spathacea* up to genus level (Duke *et al.*, 2010).

2.4 DNA Barcoding

Life on Earth is truly magnificent due to its rich diversity and endless varieties within species. According to Mora *et al.* (2011), there are approximately 8.7 million species found on Earth. Despite the richness of biodiversity is an interesting study subject for biologists, it also becomes a heavy burden for many biologists in terms of species identification (Hebert *et al.*, 2003). In the past, species identification was much relied on the recognition of morphological characteristics. This approach has four limitations as described by Hebert *et al.* (2003), namely: I) phenotypic plasticity and genetic variability may cause incorrect identification of the species; II) overlooking of morphological cryptic taxa; III) some morphological characteristics are only visible or detectable during certain stages of organisms; IV) high expertise is needed for the identification works.

Due to these limitations, a latest approach for species identification was invented, which is the DNA barcoding method. DNA barcoding is a scientific method in taxonomy that uses a relatively short genetic marker in the DNA sequence of a target organism to identify its species (Hebert *et al.*, 2003; Kress *et al.*, 2005; Lahaye *et al.*, 2008). In contrast to molecular phylogeny, it does not focus not finding the evolutionary relationships between species or varieties within a species, but rather focus on the identification of an unknown specimen whether it belongs to a documented species. In fact, DNA barcoding is very useful as it helps to assign unknown individual to a particular species and it enhances the discovery of new species (Moritz & Cicero, 2004; Kress & Erickson, 2008). For DNA barcoding works to be efficient, high level of variation must be in genetic sequence between species so that they can be differentiated from each other (Lahaye *et al.*, 2008). Besides that, the degree of variation must be low enough within species so that a clear threshold between intra- and interspecific

genetic variation can be known (Lahaye *et al.*, 2008). Next, Kress and Erickson (2008) also explained that a gene region must fulfill three characteristics to become an efficient DNA barcode, namely: I) significant species-level genetic variability and divergence; II) have conserved flanking sites for developing universal PCR primers for extensive taxonomic application; III) the length of sequence is short in order to facilitate DNA extraction and amplification works.

DNA barcoding has been a helpful method in scientific communities for the purpose of biological diversity studies. As mentioned by Schander and Willassen (2005), DNA barcoding helps to identify cryptic and polymorphic species and provides means to understand the complicated life history of unknown organism. According to Moritz and Cicero (2004), DNA barcoding should not be confused with the efforts of molecular phylogeny to resolve the 'Tree of Life'. In fact, DNA barcoding is a helpful method that provides access to new insights on biodiversity and the evolution of life on Earth (Schander & Willassen, 2005).

2.5 Internal Transcribed Spacer (ITS) Region

Internal transcribed spacer (ITS) region of nuclear ribosomal DNA have been useful in many phylogenetic studies. Feliner and Rosselló (2007) also mentioned that ITS is the most popular non-plastic target region in the nuclear genome for the purpose of evolutionary studies of various plant species. According to Feliner and Rosselló (2007), there are four reasons for the popularity of ITS region usage. Firstly, the availability of several sets of universal PCR primers to be used to work on various taxonomic groups; secondly, the multicopy structure facilitates PCR amplification even from plant specimens of herbarium; thirdly, the size of the ITS is usually moderate, that is, below 700bp, and this allows amplification and sequencing with no internal primers; fourthly, ITS also capable of providing adequate molecular markers which are suitable for evolutionary studies at the species level, due to their levels of variations (Feliner & Rosselló, 2007). ITS region is also the most often used genetic marker for distinguishing various plant species (Sun *et al.*, 2013). This is due to the high evolution rate of ITS, which is important and practical for the phylogenetic studies that involved closely-related species or individuals within a same species (Álvarez & Wendel, 2003; Feliner & Rosselló, 2007; Sun *et al.*, 2013).

The whole length of ITS sequence can be divided into two parts, namely, internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2). ITS1 is positioned between 18S and 5.8S rRNA coding sequences while ITS2 is situated between the 5.8S and 25S rRNA coding sequences (Tropp, 2008). It is a DNA region that has been found to be varying in both sequence and secondary structure in which the pattern of variation is highly correlated with taxonomic classification (Coleman, 2009). As mentioned by Coleman (2009), ITS2 region is a very useful tool as both the sequence and aspects of the secondary structure formed by initial RNA transcript can be compared. Next, ITS2 is considered to have evolved

in concert and this led to a homogenization of all copies of this gene throughout the genome (Álvarez & Wendel, 2003; Han *et al.*, 2012). Moreover, ITS2 was normally treated as a single locus in most organisms and it becomes be a suitable genetic marker for taxonomic classification (Han *et al.*, 2012). In addition, ITS2 region is considered as a good universal DNA barcode to be used in the process of identification of plant and animal species (Yao *et al.*, 2010; Chen *et al.*, 2010; Li *et al.*, 2013; Gu *et al.*, 2013). ITS2 is a good DNA barcode for plants as it exhibits several characteristics, which include the availability of conserved regions for designing universal primers, easy process of its amplification, and sufficient variability to differentiate even closely related species (Kress *et al.*, 2005; Yao *et al.*, 2010).