PHYLOGENETIC ANALYSIS OF ELEVEN MALAYSIAN RICE CULTIVARS USING A CHLOROPLASTIC DNA MARKER

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For my mom, Wahidah Binti Pohat@Puad and dad, Fauzi Bin Zainul.
No one has ever given more loving and unconditional support than you.

To my siblings, supportive friends and my dedicative supervisor.
For your courage, prayers and spiritual support to help out till this comes true.
"Mak", "Ayah" this is for you. Thank you.

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ABSTRACT

To date, both the morphological and molecular characteristics of Malaysian rice cultivars are not well documented resulting in difficulty to identify cultivars with specific origin or traits. Although some rice information are available, the information are mostly not systematically organized with limited accessibility. Thus in this work, a chloroplastic DNA (cpDNA) marker gene, the large subunit of ribulose- 1,5 - bisphosphate carboxylase (*rbcL*) gene were used to construct a phylogenetic tree of eleven Malaysian rice (*Oryza sativa* L.) cultivars. cpDNA is most commonly used in phylogenetic and barcoding study for plant species because of its high success rate in PCR amplification, appropiate length and a base substitution rate for inferring phylogeny at higher levels. The *rbcL* gene was amplified from genomic DNA of the 11 rice cultivars, cloned and sequenced. The sequences obtained was analyzed and aligned using software MEGA 6. Then, the phylogenetic analysis was constructed by using method Maximum Parsimony. The result revealed that *rbcL* gene of eleven Malaysian rice cultivars are ~1400 bp in size. Based on the tree constructed, the eleven Malaysian rice cultivars studied can be classified into two major clades. The first clade consists of MR 220 CL2, MR 219, Putih and Wai while MR 269, MR 263, MR 220, Merah Udang, Pulut Bukit, MR Q76 and Bukit Hitam falled into the second clade. Bootstrap support (BS) value in some of the branches were a bit low and these value is reflected by the small number of informative character (844 were conserved and 319 were potentially informative) to design the tree. The formation of several subclades in the tree is due to its similar genetic pattern and thus support the system classification. In overall, this study suggested that *rbcL* gene can serve as a good candidate gene to distinguish the phylogenetic relationship in Malaysian rice cultivars

ABSTRAK

Sehingga kini, ciri- ciri morfologi dan molekul oleh kultivar padi Malaysia ini tidak direkod dengan sebaiknya dan ianya mengakibatkan asal usul kultivar padi itu sukar untuk dikenalpasti. Walaupun terdapat maklumat - maklumat berkenaan padi itu, tetapi kebanyakkannya tidak disimpan secara sistematik serta sukar untuk diperoleh daripada internet. Oleh yang demikian, dalam kajian ini, satu gen penanda kloroplas iaitu unit kecil ribulosa-1,5-bisphosphat karboksilase (*rbcL*) telah digunakan untuk membuat pokok filogenetik yang terdiri daripada sebelas kultivar padi Malaysia. Kloroplas DNA secara umumnya selalu digunakan dalam kajian filogenetik dan DNA bar kod untuk spesis tumbuhan. Hal ini kerana gen tersebut mempunyai tahap kejayaan yang tinggi bagi amplifikasi PCR, panjang yang mencukupi, dan juga tahap substitusi dikalangan filogeni yang tinggi. Gen *rbcL* daripada sebelas genom DNA kultivar padi Malaysia telah diamplifikasi, diklon dan dijujuk. Jujukan yang diperolehi akan dianalisis dan disusun menggunakan perisian MEGA 6 dengan kaedah "Maximum Parsimony". Hasil analisa mendapati saiz gen *rbcL* daripada kultivar padi Malaysia adalah ±1400 bp. Berdasarkan pokok filogenetik, sebelas kultivar padi Malaysia boleh dikelaskan kepada dua kumpulan. Kumpulan pertama terdiri daripada kultivar MR 220 CL2, MR 219, Putih dan Wai sementara MR 269, MR 263, MR 220, Merah Udang, Pulut Bukit, MR Q76 dan Bukit Hitam dikelaskan kepada kumpulan kedua. Nilai bootstrap di beberapa kumpulan adalah rendah dan ini disebabkan oleh bilangan karakter yang konservatif (844 konservatif, 319 adalah karakter berpotensi). Pembentukan kumpulan atau beberapa subkumpulan adalah disebabkan oleh persaman ciri genetik, seterusnya menyokong sistem klasifikasi ini. Secara keseluruhannya, kajian ini membuktikan bahawa jujukan dari gen *rbcL* boleh digunakan untuk menentukan hubungan filogeni bagi pembezaan antara kultivar padi Malaysia.

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LIST OF ABBREVIATIONS/ SYMBOLS

±	-	plus minus
%	-	Percentage
^{o}C	-	degree Celcius
µL	-	microliter
bp	-	basepair
BS	-	bootstrap support
CI	-	consistency index
cm	-	centimetre
cpDNA	-	chloroplast DNA
g	-	gram
h	-	hour
ITS	-	Internal transcribed spacer
kb	-	kilobase
kg	-	kilogram
ng	-	nanogram
amp	-	ampicilin
LB	-	Luria- Bertani Broth
m	-	meter
min	-	minute
mL	-	mililiter
mM	-	micromolar
mP	-	Maximum Parsimony
mtDNA	-	Mitochondria DNA
nDNA	-	nuclear DNA

nm	-	Nanometer
PCR	-	Polymerase chain reaction
rbcL	-	ribulose - bisphosphate carboxylase gene
RI	-	retention index
Rpm	-	revolutions per minute
sec	-	second
T_m	-	annealing temperature
U	-	Unit
t	-	tons
v/v	-	Volume per volume
w/w	-	Weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Rice is one of the major food source of human population and serve as the second major global calories contributor after wheat (FAO, 2008). It serves as the main source of carbohydrate, providing staple food for nearly one-half of the global population (Chen *et al.*, 2004). *Oryza sativa*, which is largely cultivated in Asia, consists of two major subspecies, namely *Indica* and *Japonica*, which are believed to have been spread from China towards Southeast Asia including Malaysia (Morishima, 2001; Smith, 2003). These cultivars cover approximately 150 million hectares of land with an annual world production of around 600 million tons.

Wetland rice or lowland rice is grown on flatland with control irrigation. Rice that are planted on flatland soil is frequently flooded when seedlings are 25-30 days. Rice is widely distributed throughout the tropical, subtropical, and temperate zones of all continents worldwide and grown in submerged or waterlogged soils annually in an area approximately 77 million ha (Fageria *et al.*, 2011). Out of that, 53 % of world rice and 76 % of world's rice production comes from irrigated areas (Fageria *et al.*, 2003). Upland or aerobic rice are grown in rain-fed, naturally well-drained soils, without surface water accumulation, normally without phreatic water supply, not bunded (Fageria *et al.*, 2011). Upland rice consist of eleven percent of total global rice production and is cultivated on around 14 million ha which only has a small role in total production but yet a major food in some tropical countries

(Sohrabi *et al.,* 2013). Brazil is the largest producer of upland rice in the world. The average yield of upland rice in Brazil is about 2 t ha^{-1} which is quite low when compared to the average yield of lowland rice which normally reached about 5 t ha^{-1}. In Malaysia, upland rice are cultivated mainly in Sabah and Sarawak with a plantation area of roughly 165, 888 ha (Sohrabi *et al.,* 2013). Yields of upland rice are relatively low as compared to wetland rice and this range from 0.46 to 1.1 tonnes/hectare. Musa *et al.,* (2009) reported that research on upland rice has been neglected because of the low and unstable grain yields, although it is widely grown in the interior parts of the country. Table 1.1 shows the comparison between the upland and lowland rice cultures.

Table 1.1: Comparison between upland and lowland (wetland) rice cultivars.

Number	Lowland	Upland
1.	Cultivated on levelled, bunded, undrained soils.	Cultivated on undulating or leveled naturally drained soils
2.	Water supply through rainfall or irrigation	Water supply through rainfall
3.	Water accumulation in the field during major part of crop growth	No water accumulation during crop growth
4.	Reduced root zone during major part of crop growth	Oxidized root zone during crop growth
5.	Direct seeding or transplanting	Direct seeding
6.	Thin and shallow root system	Vigorous and deep root system
7.	High tillering	Relatively low tillering
8.	Short and thin leaves	Long and thick leaves
9.	Environmental conditions are stable and uniform	Environmental conditions are unstable and variable
10.	Incidence of diseases and insects low	Incidence of diseases and insects high
11.	Weeds are not a serious problem	Weeds are a serious problem
12.	Needs high input	Needs low input
13.	High cost of production	Low cost of production
14.	Stable and high yield	Unstable and low yield

Despite being the major staple food in Malaysia, data on characterization of local cultivars both phenotypically and genotypically is very scarce and almost inaccessible. Molecular markers technology provides powerful tools in the assessment of genetic relationship within and among the species, in which differences among the accessions can be revealed at the DNA level (Ni *et al.*, 2002; Chakravarthi and Naravaneni, 2006). This method also provides valuable information in addressing many phylogenetic questions which cannot be solved using morphological characteristics. To date, molecular systematics in plants has progressed rapidly with DNA amplification or known as polymerase chain reaction,

PCR using universal DNA barcodes mediated by direct sequencing (Schulte *et al.*, 2009; Ji *et al.*, 2008). The previous studies has claimed that large subunit of the ribulose-bisphosphate carboxylase (*rbcL*) gene is suitable for inference phylogenetic relationship at higher taxonomic levels (Schulte *et al.*, 2009). The *rbcL* gene is usually up to 1400 bp in size and the use of this gene in phylogenetic analysis and DNA barcoding has been reviewed in many studies (Spreitzer and Salvucci, 2002; CBOL Plant Working Group 2009; Wong *et al.*, 2013). This is due to its benefits as a chloroplast gene which is highly conserved and slower evolutionary rate (Hamdan *et al.*, 2013).

Till now, most information available regarding Malaysian rice research data are not systematically organized with limited accessibility. Furthermore, very little is known about the molecular genetics and morphology characters of Malaysian rice cultivars. Hence, in this study, the phylogenetic analysis of eleven Malaysian rice cultivars (*Oryza sativa* L.) was conducted using a chloroplastic marker gene known as large subunit of the ribulose-bisphosphate carboxylase *(rbcL*). The information about genetic variability at the molecular level will be useful to identify and characterize the unique germplasm that compliments the existing cultivars to aid further rice improvement in the future.

1.2 Problem Statement

To date, both the morphological and molecular characteristics of Malaysian rice cultivars are not well documented resulting in difficulty to identify cultivars with specific origin or traits. Although some rice information are available, the information are mostly not systematically organized and with limited accessibility. Thus, it is very difficult for the researchers to obtain the exact information regarding the varieties of rice being studied in relation to its origin and morphological traits. Furthermore, the exact origin of most of the rice cultivars cultivated by the local farmers are vague and unverified. The usage of DNA barcoding as biomarkers had shown promising result in confirming the origin of various plants, including rice. This method is a novel system designed to provide rapid, accurate and automatable

species identifications by using short standardized gene regions as internal species tags (Hebert and Gregory, 2005). Therefore, in this work, a chloroplastic marker, *rbcL* gene will be used to develop a systematic phylogenetic tree of eleven Malaysian rice cultivars. The analysis and information of these sequences will highlights the genetic variability at the molecular level between these cultivars and can promote further novel information regarding its evolutionary relationship and possibly be linked to other traits such as yield and resistance.

1.3 Objectives

The objectives of the study are:

(i) To extract genomic DNA (gDNA) from eleven different Malaysian rice cultivars.
(ii) To amplify and clone the chloroplastic *rbcL* gene from eleven different Malaysian rice cultivars.
(iii) To construct the phylogenetic tree of the eleven different Malaysian rice cultivars.

1.4 Scope of study

This study was carried out at Plant Biotechnology Lab, Faculty of Biosciences and Bioengineering (FBME), Universiti Tekologi Malaysia (UTM), Johor Bahru. Upland rice variety Pulut Bukit from Sibu, Sarawak and ten different wetland rice (MR 269, MR Q76, MR 263, MR 220, MR 220 CL2, MR 219 from MARDI and Wai, Putih, Bukit Hitam and Merah Udang from Sibu, Sarawak) were chosen to be used in this research. First, soil-grown plants were subjected to genomic DNA (gDNA) extraction using two methods; DNeasy ® Plant Mini Kit (Qiagen) and modified Edwards *et al.*, (1991). Then, the gDNA extracted were used as template to amplify the *rbcL* gene using *rbcL* universal and *rbcL*-rice specific

primers via PCR. Next, five purified PCR products varieties Putih, Merah Udang, Wai, Pulut Bukit and Bukit Hitam were cloned into a cloning vector (pGEM-T Easy) and sequenced while the remaining six cultivars were subjected to direct sequencing. Finally, the novel *rbcL* sequences obtained were analyzed and applied for phylogenetic tree construction of the eleven Malaysian rice cultivars using MEGA 6 software via Maximum Parsimony method.

1.5 Significance of the study

In this study, gene of the large subunit of ribulose–bisphosphate carboxylase (*rbcL*) was used as an alternative approach for morphology identification and to study the evolutionary status and relationship among eleven Malaysian rice cultivars. At the end of this study, the relationship between all eleven Malaysian rice cultivars will be determined using the phylogenetic analysis constructed from the novel *rbcL* sequences. From this finding, the relationship pattern among all eleven cultivars could be used as source of knowledge for successful interbreeding on creating new cultivars in the future.

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