MOLECULAR CHARACTERIZATION OF LOCAL RICE (*Oryza sativa* L.) GENOTYPES FROM YANGON REGION IN MYANMAR USING SSR MARKERS

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NOVEMBER 2019

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A Thesis Submitted to the Post-Graduate Committee of the Yezin Agricultural University in Partial Fulfillment of the Requirements for the Degree of Master of Agricultural Science in Molecular Biology and Biotechnology

Division of New Genetics,
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Nay Pyi Taw, Myanmar

NOVEMBER 2019

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DECLARATION OF ORIGINALITY

This thesis represents the original works of the author, except where otherwise stated. It has not been submitted previously for a degree at any other University.

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DEDICATED TO MY BELOVED PARENTS

U MAUNG AND DAW DAUNG

ACKNOWLEDGEMENTS

Firstly, I would like to express my heartfelt thanks to Dr. Nang Hseng Hom, Rector, Yezin Agricultural University (YAU), Dr. Ye Tint Tun, Director General, Department of Agriculture (DOA), Ministry of Agriculture, Livestock and Irrigation for their kind permission and encouragement to conduct this study, Dr. Shanti Lal Mehta, Former Resident Advisor, Dr. Ram Krishna Pal, Resident Advisor, Advanced Center for Agricultural Research and Education (ACARE), Dr. Soe Soe Thein, Pro-rector (Academic Affair) and Dr. Kyaw Kyaw Win, Pro-rector (Administration), Yezin Agricultural University (YAU), for their kind permission, invaluable suggestions and kind administrative support to this study.

My special thanks go to U Myint Swe, Director, Coffee and Seasonal Crop Department, Department of Agriculture (DOA) for his permission to conduct the Master Degree Program and encouragement throughout the study.

I would like to express my highly gratitude and deepest appreciation to my beloved supervisor, Dr. Nyo Mar Htwe, Deputy Director and Head, Division of Capacity Building, Advanced Center for Agricultural Research and Education, YAU, for her inspiring guidance and suggestions, invaluable overall supervision, generous helps, motherly concern, encouragement, and understanding to me throughout the research.

My specially gratitude to Dr. Than Myint Htun, member of supervisory committee, Deputy Director and Head, Division of New Genetics, Advanced Center for Agricultural Research and Education, YAU for his scholarly guidance, encouragement, comments, and invaluable suggestions to my research and preparation of this manuscript.

I wish to extend my special thanks to Dr. Su Latt Phyu, member of supervisory committee, Lecturer, Department of Plant Breeding, Physiology and Ecology, Yezin Agricultural University for her valuable suggestions, comments and guidance in the preparation of thesis.

I would like to express my highly gratitude and deepest appreciation to my supervisory committee member, Dr. Akshay Talukdar Principle Scientist & Project Associate, ICAR-IARI Indian Agricultural Research Institute, New Dehli for his valuable suggestions, generous helps, encouragements, and thesis preparation.

I am deeply grateful to my external examiner, Dr. Min San Thein, Deputy Director and Head, Seed Bank, Department of Agricultural Research (DAR) for his

valuable critics and comments to my manuscripts, encouragement and kind support throughout the duration of my study period.

I am grateful to my teacher, Dr. Moe Kyaw Thu, Lecturer, Department of Agricultural Biotechnology, Yezin Agricultural University for his valuable critics and comments to my manuscripts, encouragement and kind support throughout the duration of my study period.

Thanks, are also due to Daw Me Me Aung, Daw Hsu Yi Mon, Daw Yee Phyo Mon, Daw Thi Thi Myint, Daw Cho Witt Yee Soe, my friends and staffs of Advanced Center for Agricultural Research and Education, YAU for their support to complete my study successfully. I would also like to express my special thanks to all my friends, seniors and juniors who were being with me, provided numerous supports, social encouragement, technical helps, guidance and assistance during my study program.

Finally, most special thanks should go to my beloved wife, Daw Aye Yamoan and my lovely son, Mg Chan Nyein Zaw, and my lovely daughter, Ma Shoon Lae Lae Zaw for their encouragement, moral and financial support, patience and understanding during my study program.

ABSTRACT

In Asia, Myanmar is center of rice genetic diversity as there are numerous rice varieties under diverse agro-ecological zones. In order to estimate genetic diversity of rice germplasm in Yangon region of Myanmar, 102 genotypes from different parts of region were analyzed by using 12 SSR markers at New Genetics laboratory, Advanced Center for Agricultural Research and Education (ACARE) in Yezin Agricultural University (YAU). In SSR characterization, the maximum and minimum Polymorphic Information Content (PIC) values were found to be 0.79 and 0.21 for the primers RM229 and RM201, respectively. The mean value of PIC was 0.69. The total number of alleles was 91 and the average number of alleles per locus was 7.58. Average genetic diversity was 0.72 indicating high genetic diversity among the genotypes. Phylogenetic cluster analysis of SSR data based on UPGMA and Nei's genetic distance divided into eight groups. These results reflect the high genetic differentiation existing in rice germplasm. The population structure analysis in the present study resulted in nine population. This result indicated that SSR markers have proved to be useful for detecting genetic diversity of Yangon rice genotypes, and the occurrence of a considerably high number of rare and unique alleles in the genotypes indicates their potentiality as a reservoir of rare genotypes for use. The results from molecular data can be the integral part of plant breeding program, particularly, the development of new rice varieties with desirable qualities and also useful for plant varietal protection.

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CHAPTER I
INTRODUCTION

Most of the world's rice is cultivated as daily diet in Asia, which constitutes more than half of the global population (Rahman, Molla, Alam, & Lutfur, 2009). Rice (*Oryza sativa* L.) genetic resources are widely available in the worldwide (Chakravarthi & Naravaneni, 2006). Myanmar is one of the centers of rice genetic resources in Asia which spreads along the Himalayas from Iran to Myanmar consisting of very diverse varieties (Glaszmann, 1986). Many local varieties have been grown in Myanmar for centuries. The areas including Assam in India, Myanmar, Laos and Yunnan in China could provide the richest spectrum of rice varietal diversity (Chang, 1976). For a long time, landraces, traditional and local rice varieties in Myanmar are grown over the whole country, under diverse agro-ecological zones such as flood-prone area, rain fed lowland, upland, and hilly regions.

Development of new biotechnological techniques provides increased support to evaluate genetic variation in both phenotypic and genotypic levels. Molecular markers are powerful tools in the assessment of genetic variation, in the elucidation of genetic relationships within and among species and have demonstrated the potential to detect genetic diversity and to aid in the management of plant genetic resources (Da Silva, 2005, Song et. al., 2003, Virk, Newbury, Jackson, & Ford-Lloyd, 2000)

Genetic divergence among the genotypes plays an important role in the selection of parents having wider variability for different characters. Genetic diversity can be evaluated with morphological traits, seed proteins, isozymes and DNA markers. In contrast to morphological traits, molecular markers can reveal abundant difference among genotypes at the DNA level, providing a more direct, reliable and efficient tool for germplasm characterization, conservation, management and untouched by environmental influence (Xu, Wang, Zheng, & Wang, 1974). Molecular markers have been widely used to study the genetic variation and diversity of breeding materials, which were less influenced by temporal, spatial and environmental conditions (Hashimoto et al., 2004). Xu et al. (1974) also presented as molecular marker technology is the powerful tool for determining genetic variation in rice varieties.

In rice, molecular markers have been used to identify accessions (Olufowote et al., 1997; P. S. Virk, Ford-Lloyd, Jackson, & Newbury, 1995), to determine the genetic structure and pattern of diversity for cultivars of interest (Thu et al., 2011; Zhang,

Maroof, Lu, & Shen, 1992) and to optimize the assembly of core collections (Schoen & Brown, 1995). Among various PCR based markers, SSR markers are more popular in rice because they are highly informative, mostly monolocus, codominant, easily analyzed and cost effective (Garcia et al., 2004). Microsatellite markers detect a significantly higher degree of polymorphism in rice and are especially suitable to evaluate the genetic diversity among closely related rice cultivars (Miah et al., 2013). In rice, SSR markers are distributed relatively uniformly through the genome and have been detected a high level of allelic diversity in genotypes and distantly related species (McCouch et al., 2001). Genetic divergence among the genotypes plays an important role in the selection of parents having wider variability for different characters. The identification of rice genotypes and their inter-relationships is essential and it can be done by molecular markers.

In Myanmar, the classical way of assessing genetic diversity has been utilized on the study of morpho-agronomic variability and importance for the utilization in rice improvement program. Genetic variation by molecular marker is not yet well developed in Myanmar. Therefore, the present study was carried out by SSR markers to assess the genetic diversity of rice genotypes from Yangon Region in Myanmar and to examine the population structure among genotypes from Yangon Region using model-based approach.

CHAPTER II
LITERATURE REVIEW

Rice (*Oryza sativa* L.) is a plant belonging to the kingdom plantae, division magnoliophyta, class liliopsida, order cyperales, family gramineae, genus *Oryza* and species *sativa*. (Brar, 2003). The species *sativa* is made up of three subspecies namely: Indica (tropical and subtropical distribution), Javanica (grown in Indonesia) and Japonica (temperate distribution). Indica and Japonica are the two most widely grown and researched ecotypes (Olufowote et al., 1997). It has a relatively small genome size which makes it an ideal model plant for study of grass genetics and genome organization (Causse et al., 1994). Due to its importance as a food crop, it is planted on approximately 11% of the Earth's cultivated land area (Khush, 2005).

Rice domestication has led to development of a large number of *Oryza sativa* cultivars. Around 100, 000 different rice genotypes are held at the international rice gene bank of which most are *Oryza sativa* varieties. Rice diversity based on morphology and quality traits such as grain size, shape, colour, aroma and starch content has been increased due to different socio-cultural traditions and practices (Khush, 1997).

2.1 Genome Evolution of Rice

Rice genome has vast variation more than thousand varieties have been produced around the world (Ashfaq, Haider, Khan, & Allah, 2012). The genus *Oryza* has 12 chromosomes in its genome. *Oryza sativa, O. glaberrima* and 14 wild species are diploid (2n=24) with 24 chromosomes and relatively small genome size (430 million base pairs). Other 7 wild species are tetraploids (2n=48) with 48 chromosomes (Brar, 2003) as shown in Table 2.1.

Genome analysis done on the basis of chromosome pairing behavior and fertility in interspecific hybrids and degree of sexual compatibility has made possible to classify species in the genius *Oryza* into nine distinct genomes. The genus *Oryza* has also been classified into four complexes which reflect genetic diversity of genus (Khush, 1997). The four *O. sativa* complex species are all diploid and they have AA-type genomes and nine are pantropical. Of these four complexes, *O. sativa* and *O. Officinalis* complexes are the best studied for characterization. The *sativa* complex comprises the cultivated species *O. sativa* and *O. glaberrima* and their wild ancestors' viz., perennial rhizomatous *O. longistaminata*, *O. barthii* (formerly *O. breviligulata*) and *O.*

Table 2.1 Species complexes of the genus Oryza

Species Complex	Chromosome number	Geographical distribution
I. *sativa* complex	**24**	
O. sativa L.	24	Worldwide: originally South and Southeast Asia
O. nivara	24	South and Southeast Asia
O. rufipogon	24	South and Southeast Asia, South China
O. meridionalis	24	Tropical Australia
O. glumaepetula	24	Tropical America
O. glaberrima	24	Tropical West Africa
O. barthii	24	West Africa
O. longistaminata	24	Tropical Africa
II. *officinalis* Complex		
O. punctata	24	East Africa
O. rhizomatis	24	Sri Lanka
O. minuta	48	Philippines, New Guinea
O. malamphuzaensis	48	Kerala and Tamil Nadu
O. officinalis	24	South and Southeast Asia
O. alta	48	Central and South America
O. grandiglumis	48	South America
III. *meyeriana* Complex		
O. granulata	24	South and Southeast Asia
O. meyeriana	24	Southeast Asia
IV. *ridleyi* Complex		
O. longiglumis	48	Indonesia, New Guinea
O. ridleyi	48	Southeast Asia
V. unclassified (belonging to no complex)		
O. brachyantha	24	West and Central Africa
O. schlechteri	48	Indonesia, New Guinea

Source: (Brar, 2003)

rufipogon, *O. nivara* and *O. sativa f. spontanea*. The species of *sativa* complex constitute the primary gene pool of rice while the species belonging to *Officinalis* complex constitute the secondary gene pool. The species belonging to *Meyeriana*, *Ridleyi* complexes and *O. schlechteri* constitutes the tertiary gene pool (Khush, 1997).

The total length of rice nuclear genome was calculated to be 388.8Mb of DNA distributed among the 12 chromosome pairs, and included the genes that encode some 38,000 proteins (Mausa, 2014). The genome also consists of one pair of circular mitochondrial DNA, and a circular chloroplast DNA (Paterson, Freeling, & Sasaki, 2005).

2.2 Geographical Origin

The centers of origin and diversity of *O. sativa* and *O. glaberrima* have been traced using archaeological evidences, geographical distribution and genetic diversity. River valleys of Yangtze and Mekon are the primary centers of origin of *O. sativa*. On the other hand, Niger River delta in Africa is the centre of origin of *O. glaberrima* (Huang et al., 2012). The foothills of the Himalayas, northern parts of Myanmar and Yunnan Province of China are some of the centres of diversity for Asian rice species. A large number of *O. sativa* cultivars has been developed through centuries of rice domestication either through natural evolution, conventional breeding or marker assisted breeding (MAB).

Around 100,000 different rice genotypes are held at the international rice gene bank in Philippine. The need for increasing rice cultivation depends not only on cultural or traditional practices, but also, on their inbuilt genetic potential to withstand stresses. A successful breeding program will depend on the genetic variability of a crop for achieving the goals of improving the crop and producing high yielding varieties (Padulosi, 1993). Both phenotypic and genetic diversity of *Oryza sativa* is very wide. Different rice varieties are distinguished based on many features such as growth habit, adaptation to different water regimes, shape, size and color of the grain among many others. The first step in achieving is to evaluate and characterize available rice germplasm or genotypes at both morphological and molecular levels; as phenotypic and genotypic diversity will reveal important traits or accessions of interest to plant breeders (Singh, 1989).

2.3 Genetic Diversity in Rice

Genetic diversity may be also gauged using morphological, and biochemical characterization and evaluation: Each technique has its particular bottlenecks / problems / limitations:

1. Morphological characterization does not require expensive technology but large tracts of land are often required for these experiments, making it possibly more expensive than molecular assessment.
2. Biochemical analysis is based on the separation of proteins into specific banding patterns. It is a fast method which requires only small amounts of biological material. However, only a limited number of enzymes are available and thus, the resolution of diversity is limited.
3. Molecular analysis comprises a large variety of DNA markers, which can be employed for analysis of variation. Different markers have different genetic qualities (they can be dominant or co-dominant, can amplify anonymous or characterized loci, can contain expressed or non-expressed sequences, etc.). However, the methods require expertise and high initial investment.

Asian cultivated rice (*Oryza sativa*. L) is normally classified into two subspecies; indica and japonica. Indica-type landraces predominates in Myanmar as 81 percentage of total landraces (Khush et al., 2003) and Japonica-type landraces specially influence in Eastern region (Saw, Doi, Aye, Irie, & Yoshimura, 2006). However, landrace diversity and its dissemination depend on the adaptation to local agro-ecology, socio-economic and environmental harsh conditions (biotic and abiotic stress). A wide range of vernacular names for each variety does not always mean to represent genetically diversified cultivars because of linguistic differences among different ethnic groups (Watanabe et al., 2007). If the farmer-named varieties are not genetically distinct, farmer taxonomies and nomenclature which are typically localized and culturally determined would not affect in identification and qualification of diversity in agricultural ecosystem consistency in naming and distinguishing landraces is essential in evaluation of diversity and socio-economic studies (Jarvis et al., 2007). Myanmar farmers classify rice with some standards; growth duration, water regime and seed size. Actually, agro-morphological trait is important for farmers because they identify or distinguish high yield varieties for growing every year. The farmer chooses to plant a particular variety because of its desirable characteristics.

Study of genetic diversity is the process by which variation among individuals or groups of individuals or populations is analyzed by a specific method or a combination of methods (Mohammedi, 2010). Data sets consisting of measurements of several attributes (biochemical, morphological and agronomic characters) for various accessions are necessary for assessing genetic diversity and classification. Diverse data sets have been used to analyze genetic diversity in crop plants; such data sets are passport data and morphological data (Smith, Smith, & Wall, 1991).

Genetic variability is the mainstay for the success of any breeding program, hence assessing the level of genetic diversity among the rice varieties or genotypes has been of great interest to the breeders. Rice taxonomists are interested in the rapid classification of different taxonomic groups, while breeders are concerned about the determination of usable agronomic variation in breeding programs (Zhou, Xie, & Ge, 2003). Although large number of them is available, little analysis of the genetic diversity has been done at molecular level so far. Genetic diversity studies based on molecular markers generate information-base for more efficient use of the valuable genetic resources. Pre-breeding or genetic enhancement has become a necessary and planned part of all rice breeding activities.

Detailed evaluation and characterization of available rice genotypes is one of the main prerequisites in conservation and sustainable utilization of rice genetic resources. This ensures that maximum variation is captured in designing breeding strategies aimed at increasing productivity. Information on diversity and population structure is expected to assist plant breeders in selection of parents to be used in hybridization programmes, provide a more rational basis for expanding the gene pool and for identifying plant materials that harbor more valuable alleles for genetic improvement (Semon, Nielsen, Jones, & McCouch, 2005). Therefore, rice diversity is the foundation for variety improvement programmes.

Better use of genetic diversity can create new rice cultivars that resilient to both current biotic and abiotic production stresses as well as achieve high yield. Almost in all major crop species, morphological and physiological descriptors are available to establish the uniqueness of a variety (Moukoumbi et al., 2011). Hence, characterization and identification of rice cultivars are crucial for the genetic varietal improvement, release and seed production programmes.

In order to select resistant and highly productive varieties, it is required an assessment of diversity at some level. The assessment of genetic diversity within and

between populations is routinely performed at the molecular level using various laboratory-based techniques such as allozyme or DNA analysis, which measure levels of variation directly, (Barcaccia, 2010),

2.4 Molecular Markers and Applications

A molecular marker is defined as a particular segment of DNA (Deoxyribonucleic acid) that is representative of the differences at the genome level, readily detected and whose inheritance can easily be monitored. It can also be defined as a genomic locus, detected through probe or specific starters (primer) which, by virtue of its presence, distinguishes unequivocally the chromosomic trait which it represents as well as the flanking regions at the 3' and 5' extremity (Barcaccia, 2010).

Molecular markers such as isozyme, Restriction Fragment Length Polymorphism, Amplified Fragment Length Polymorphism, Random Amplified Polymorphic DNA, Simple Sequence Repeats, Inter Simple Sequence Repeats and Single Nucleotide Polymorphism have a number of applications in the study of plant genetics. They are commonly used when studying rice genetic diversity because they are highly informative, mostly mono locus, co-dominant and affordable (Chambers & Avoy, 2000). Advances in plant genetics and molecular biology have led to the development of many types of molecular markers that can be used to characterize germplasm. Different types of DNA markers are available, each method differing in principle, application, type, and amount of polymorphism detected, cost, and requirement (Jackson, 1997).

DNA markers that differentiate genotype are more reliable and convenient than physiological characters in the identification and characterization of genetic variation (Zeng et al., 2004). Seetharam et al., (2009) concluded that, the best measure to analyze genetic diversity among genotypes would be with the use of all information; both from morphological characters and DNA based markers.

2.5 Microsatellites, Simple Sequence Repeats Markers

Simple sequence repeats (SSR) markers are di-, tri-, and tetra-nucleotide tandem repeats containing loci of eukaryotic genomes. These are actually non-coding regions which remained conserved during the course of evolution and are ideal for DNA fingerprinting and varietal identification. They are valuable genetic markers which can detect high level of allelic diversity based on the variability in the tandem repeats in the

core unit and are co- dominant. It is demonstrated that these loci are very polymorphic due to changes in the number of repeating units among the individuals of populations. Each SSR locus can easily be amplified by using PCR knowing the DNA sequence flanking the repeat region specifically.

Microsatellites are also called simple sequence repeats (SSRs) and, occasionally, sequence-tagged microsatellite sites (STMS) or simple sequence repeat polymorphisms (SSRPs). SSR markers vary widely among organisms as high as 50 copies of the repeated unit. One of the distinguishing features of SSR loci is their hyper variability, which is associated with the expansion/contraction potential of the SSR motif itself (Chen et al., 2002). Mendelian inheritance, high allelic variation, with genome-wide distribution (Garza & Freimer., 1995; Chen et al., 2002) mentioned that the variability of SSR loci at the sequence level, undertaken in a range of species have revealed numerous instances of size homoplasy, where alleles with the same molecular weight contain different internal mutations. The mutation involves insertions, deletions and base substitutions in both the microsatellite motif and its flanking regions (Grimaldi & Crouau-Roy., 1997; Peakall et al., 1998; Rose and Falush, 1998: Chen et al., 2002).

Microsatellite or SSR markers have been developed in many species for the analysis of genetic diversity, because they tend to be multi-allelic and consequentially highly informative in most populations (Gealy, Agrama, & Eizenga, 2009). The level of polymorphism at microsatellite loci can be expressed in terms of the number of alleles and the expected heterozygosity in gene diversity (Innan, Terauchi, & Miyashita, 1997) while the measure of polymorphism depends on the length of DNA fragment or repeats (Fayed & Abbas, 2016). Conventional microsatellite marker analysis based on the evaluation of amplicon size variation is efficient and inexpensive, enabling discovery of polymorphisms throughout the genome (Jun Yu et al., 2002). While this approach is useful for examining genetic variation among individuals derived from biparental mating or within a species or among closely related species, the homoplastic nature of microsatellite markers suggests that DNA sequencing of amplified fragments is necessary when one wishes to obtain reliable information as the basis for phylogenetic reconstruction or analysis of genetically diverse accessions (Garza, Slatkin, & Freimer, 1995; Svebak, Martin, Svebak, & Apter, 1997).

The limiting feature of the application of these markers is the need for prior sequence information for developing primers for locus-specific PCR amplification. Generation of complex banding patterns for SSR loci could be due to various reasons

such as type of repeat, non-optimization of PCR conditions and the nature of genome. SSR were chosen for the analysis of genetic diversity of rice landraces and wild relatives because several works have shown that these markers are very powerful for differentiating individual germplasm accessions, particularly when they are closely related (Murray-Bligh, 1999; Xu et al., 2004; Jeung et al., 2005).

2.6 Molecular Characterization with SSR markers in Rice

The SSR markers have been increasingly applied in rice germplasm. Upadhyay, Singh, and Neeraja (2011) studied genetic diversity of popular of 29 rice varieties in India using 12 SSR markers and identified genotype specific alleles in 14 popular rice varieties. Ghneim Herrera et al. (2008) assessed genetic diversity in Venezuelan rice cultivars using simple sequence repeat markers to broaden the genetic bases of rice germplasm. Furthermore, Brondani, Borba, Rangel, and Brondani (2006) characterized the allelic diversity of 192 traditional varieties of Brazilian rice using 12 simple sequence repeat markers. It revealed identical accessions with the same name, few with different names and a mixture of pure lines, indicating that SSR markers are fundamental to determining the genetic relationship between landraces. Mausa., (2010) used SSR markers to analyze 70 samples of Tanzanian rice landraces, showed no significant genetic variation among the landraces studied.

In Myanmar, (Watanabe et al., 2016) studied the genetic diversity of 175 accessions (including landraces and improved types) from upland and lowland ecosystems using 65 microsatellites markers, with average polymorphism information content (PIC) value of 0.82 per locus ranging from 0.519 to 0.919. Variation of genetic structure in 38 Pawsan cultivars from Ayeyarwaddy region in Myanmar was documented to conserve and exploit potential cultivars for breeding program (Min et al., 2014). It indicated that pair-wise of genetic similarity of Pawsan cultivars ranged from 14% - 84% and averaged of 49% in SSR genotyping. Aung (2007) also reported that SSR analysis in variation among Myanmar rice varieties, in which the average alleles per locus and the average gene diversity for the Myanmar rice varieties were 5.94 and 0.70.

2.7 Population Structure in Rice

Population used in association mapping should possess as many phenotypes as possible (Flint-Garcia et al., 2005). Linkage disequilibrium (LD) is the non-random co-

segregation of alleles at two or more loci. The abundant variation of the rice collection provides an important reservoir of genetic diversity and potential sources of beneficial alleles for rice breeding. However, to the knowledge, no earlier research is available to apply association mapping in a core collection, which might be due to lack of information on the population structure and LD of the core collection populations.

Population structure is an important component in association mapping analysis because it can reduce both type I and II errors between molecular markers and traits of interest in an inbreeding species (Flint-Garcia, Thornsberry, & Buckler IV, 2003; Goldstein & Weale, 2001; Agrama, Eizenga, & Yan, 2007; Agrama & Eizenga, 2008). The presence of subpopulations can result in spurious associations due to confounding of unlinked markers with phenotypic variation (Buckler IV & Thornsberry, 2002). Low level of LD could lead to impractical whole-genome scanning because of the excessive number of markers required (Kruglyak, 1999). The resolution of association studies in a test sample depends on the structure of LD across the genome (Remington et al., 2001). Therefore, information about the population structure and extent of LD within the population is of fundamental importance for association mapping.

Several previous researches on rice population structure have been reported. Five major groups, i.e. indica, aus, aromatic, temperate japonica, and tropical japonica were detected in a sample of 234 rice varieties (Garris et al., 2005). Eight subpopulations were found corresponding to major geographic regions among 103 rice accessions (Agrama et al., 2007). Seven subpopulations were detected within rice landrace in Guizhou province, China (Zhang et al., 2007). Two subgroups including indica and japonica as well as six sub-subgroups were found within a primary rice core collection (Zhang et al., 2009). Seven subgroups were found within a 416 rice population (Jin et al., 2010). The varied numbers of subgroup might be due to different methods, different numbers of marker, different rice populations applied in population structure examination, which should be further studied. However, no information on the population structure of a rice core collection assessed with a large SSR marker set was available. Furthermore, no information is available on the number of SSRs required for such analyses.

CHAPTER III
MATERIALS AND METHODS

3.1 Plant Materials

A total of 102 rice genotypes were used in this study. All these accessions were obtained from Seed Bank, Department of Agricultural Research (DAR), Ministry of Agriculture, Livestock and Irrigation (MoALI), Republic of the Union of Myanmar. A detailed description of rice genotypes is shown in Appendix (1).

3.2 DNA Extraction

Rice genomic DNA was extracted from 21-days-old seedling leaves collected from each accessions, by simple DNA extraction method (Isoproponol precipitation method) which is shown in Appendix (2).

3.3 DNA Quality Control

DNA quantification was done in two methods such as (a) Gel Electrophoresis and (b) Nano drop Spectrophotometer method.

(a) Gel Electrophoresis

The quality of the genomic DNA was determined in a 1% agarose gel in 100ml TBE electrophoresis by running 10 µl of genomic DNA at a voltage of 75 for 45 minutes. Gels were viewed under UV illumination and photographed using GDS 8000 Gel Documentation System (UVP Inc., California, and USA).

(b) Nano Drop Spectrophotometer Method

After extraction, the purity and concentration of the extracted DNA were determined using a spectrophotometer (Thermo Scientific Nano drop 2000 system). The DNA quality was assessed using the absorbance ratio at 260 to that at 280 nm wavelengths (A260/A280).

3.4 PCR Amplification Using SSR Marker

Markers were chosen according to their location on the rice genetic map and their suitability for high-throughput genotyping. Twelve SSR markers distributed on the 12 chromosomes were employed to analyze population structure (Table 3.1). SSR markers information is available in GRAMENE (http://www.gramene.org). The polymerase chain reaction (PCR) was performed in a total volume of 25µl per reactio

Table 3.1 Sequence information and repeat motif of twelve SSR markers used in this study

Marker	Chr.	Forward Primer	Reverse Primer	Repeat Motif	Annealing Temperature
RM237	1	CAAATCCCGACTGCTGTCC	TGGGAAGAGAGCACTACAGC	(CT)18	55
RM208	2	TCTGCAAGCCTTGTCTGATG	TCTGCAAGCCTTGTCTGATG	(CT)17	55
RM60	3	AGTCCCATGTTCCACTTCCG	ATGGCTACTGCCTGTACTAC	(AATT)5AATCT(AATT)	55
RM241	4	GAGCCAAATAAGATCGCTGA	TGCAAGCAGCAGATTTAGTG	(CT)31	55
RM31	5	GATCACGATCCACTGGAGCT	AAGTCCATTACTCTCCTCCC	(GA)15	55
RM225	6	TGCCCATATGGTCTGGATG	GAAAGTGGATCAGGAAGGC	(CT)18	55
1RM2	7	ACGTGTCACCGCTTCCT	ATGTCCGGGATCTCATCG	(GA)13	55
RM44	8	ACGGGCAATCCGAACAACC	TCGGGAAAACCTACCCTACC	(GA)16	55
RM201	9	CTCGTTTATTACCTACAGTACC	CTACCTCCTTTCTAGACCGATA	(CT)17	55
RM258	10	TGCTGTATGTAGCTCGCACC	TGGCCTTTAAAGCTGTCGC	(GA)21(GGA)3	55
RM229	11	CACTCACACGAACGACTGAC	CGCAGGTTCTTGTGAAATGT	(TC)11(CT)5(C)3(CT)5	55
RM247	12	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG	(CT)16	55

containing 2.5µl of template DNA (5ng / µl), 1µl of each forward and reverse primers, 1.25µl dNTPs (10mM), 0.2µl Taq polymerase (5 U/µl), 0.3µl of $MgCl_2$ (50 mM) and 2.5µl 10×PCR buffer. The PCR amplification was carried out on a thermal cycler at an initial temperature of 94°C for 5min, followed by 35 cycle of 1min denaturation at 94°C, 55°C for 30s (primer annealing occurred with most of the primers while some were adjusted), 1 min extension at 72°C with a final extension for 7 min at 72°C and then stored at 4°C.

3.5 Agarose Gel Electrophoresis

The PCR products were separated using horizontal agarose gel electrophoresis. The amplified DNA fragments were separated on 3% agarose gel stained with 0.5mg/ml ethidium bromide. After that 2µl loading dye (6x Bromophenol blue) was added to the PCR products. When the gel was set, the PCR products were added in the wells submerged in 1X TBE buffer. The samples were then run at 120 volts for 2 hours, then observed on UVPRO (Uvipro Platinum, EU) gel documentation unit.

3.6 Genotype Score and Data Analysis

For each marker, alleles for the data set were scored according to size of base pairs of the 100bp ladder DNA marker. All alleles were scored with the smallest-largest-sized alleles representing the start of the first scoring and end of the last scoring respectively. Genetic similarities were estimated from the matrix of binary data using jacquard similarity coefficient. To infer genetic relationships and phylogeny, the similarity coefficients were used for cluster analysis of the rice cultivars utilizing the complete linkage method. Basic statistics were calculated using the genetic analysis package Power Marker V 3.23 (Liu & Muse, 2009) or diversity measurements at each microsatellite locus, including the total number of alleles (NA), allele frequency, major allele frequency (MAF), gene diversity (GD), polymorphism information content (PIC) and dendrogram was viewed by MEGA 6 program software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013)

For the analysis of population structure, the model-based software program, Structure version 2.3.1 software (Pritchard, Stephens, & Donnelly, 2000) was used. In this model, a number of populations (K) are assumed to be present, each of which is characterized by a set of allele frequencies at each locus. Individuals in the sample are assigned to populations (groups), or jointly to more populations if their genotypes indicate that they are admixed. All loci are assumed to be independent, and K

population is assumed to follow Hardy-Weinberg equilibrium. The posterior probabilities were estimated using a Markov Chain Monte Carlo (MCMC) method. The MCMC chains were run at burn-in period lengths 10,000 at fixed iterations of 5 for each fixed number of population (K) from one to ten.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Allelic Polymorphism

The 12 SSR primers were used across 102 rice accessions. These SSR markers identified 91 alleles across all rice accessions. The allelic richness per locus varied widely among the markers, ranging from 5 to 11 alleles, with an average of 7.58 alleles per locus (Table 4.1). The highest number of alleles (11) was detected in the marker RM 44 and the lowest number of alleles (5) was detected in the markers RM 201 and RM 237. The average number of alleles per locus obtained in the present study was less than that reported in previous study by Kuroda, Sato, Bounphanousay, Kono, and Tanaka (2007). The difference in average allele per locus might be due to diverse nature of genotypes used by different researchers and selection of SSR markers. Rice accessions shared a common major allele at 12 loci from 0.24 (RM 225) to 0.88 (RM 201). A moderate level of allele frequency exists in these loci of rice accessions with the average 0.40 (Table 4.1). Similar results were also reported by Prabakaran, Paramasivam, Rajesh, and Rajarajan (2010) when investigated genetic variability of 12 rice landraces of India using five primers. However, this value is quite low compared with those reported in other places of the collections. Rahman, Sohag,& Rahman, (2010) reported that the number of alleles per locus varied from 3 to 8, with an average number of alleles per locus at 4.86. Mausa, (2014) reported a range of 6 to 22 numbers of alleles per primer with an average of 14.7 alleles per locus and this indicates a less magnitude of diversity with reference to the fewer markers among the plant materials. Information available on these alleles present in different genotypes will be very useful for developing the mapping populations for genome analysis as well as in applied breeding programs. Molecular-based biological and geographical diversity differed with respect to allelic richness, frequency of rare alleles, the common and most frequent alleles, and group-specific unique alleles.

According to Ni et al., (2002) the result showed that rare alleles (frequency < 0.05) comprised 39.56%, whereas intermediate (frequency 0.1-0.5) comprised 60.44% and there were no abundant alleles (frequency > 0.5) (Figure 4.1). Out of 12 polymorphic loci, only two markers RM 44 and RM 247 provided the highest number of 7 rare alleles, followed by RM 258 (6) and RM 241 (4), RM 201 (3), RM 2 (2), RM 229 (2), RM 225 (2), RM 208 (1), RM 31 (1) and RM 237 (1).

Table 4.1 Number of alleles, polymorphic information content and genetic diversity index for 12 simple sequence repeat (SSR) loci in the 102 accessions.

Marker	Chr. No	No. of observation	MAF	NA	RA	GD	PIC
RM 237	1	91.00	0.46	5	1	0.65	0.59
RM 208	2	102.00	0.46	6	1	0.70	0.66
RM 60	3	102.00	0.26	6	0	0.80	0.77
RM 241	4	102.00	0.34	9	4	0.76	0.72
RM 31	5	99.00	0.40	7	1	0.76	0.74
RM 225	6	102.00	0.24	7	2	0.80	0.77
RM 2	7	101.00	0.39	7	2	0.76	0.73
RM 44	8	101.00	0.32	11	7	0.80	0.78
RM 201	9	101.00	0.88	5	3	0.22	0.21
RM 258	10	102.00	0.32	10	6	0.79	0.76
RM 229	11	102.00	0.26	8	2	0.81	0.79
RM 247	12	94.00	0.40	10	7	0.75	0.71
Mean		99.92	0.40	7.58		0.72	0.69
Total				91	36		

MAF=Major Allele Frequency, NA=Number of alleles, Rare allele (RA) =Number of alleles that frequency < 0.05, GD= Gene Diversity, PIC= Polymorphic Information Content.

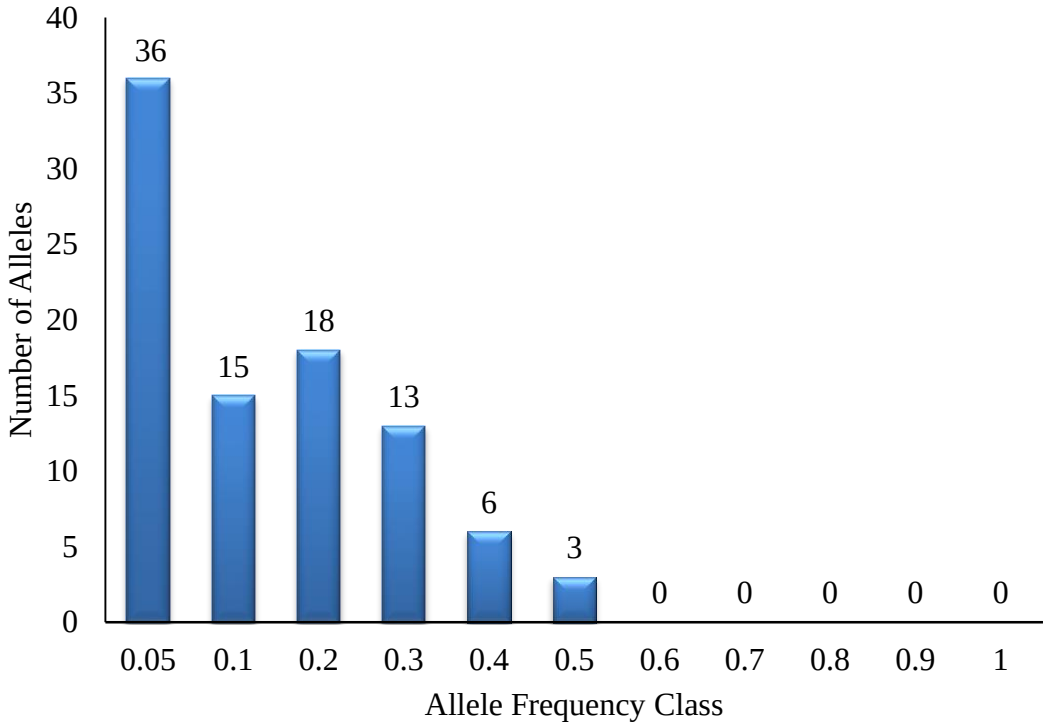


Figure 4.1. Histogram of allele frequencies for all 91 alleles in the 102 rice accessions.

4.2. Polymorphic Information Content

In the present research, the degree of polymorphism detected by 12 loci markers could not be correlated with number of allele and Polymorphic Information Content (PIC) values. Certain loci producing similar number of alleles were not significantly different in their PIC values. For example, 5 alleles were detected at each of the two loci, RM 201 and RM 237, 6 alleles were detected at each of the two loci, RM 60 and RM 208, 7 alleles were found at each of the three loci RM 225, RM 31 and RM 2, and 10 alleles were at RM 258 and RM 247, no significant difference was detected in their PIC values. The number of alleles detected at RM 229 locus for all genotypes were less than other loci (RM 241, RM 258 and RM 247 and RM 44), and this marker had the highest values of PIC (Table 4.1). Thein et al., (2012) also found no significant difference between number of alleles and PIC values.

PIC values ranged from 0.21 (RM 201) to 0.79 (RM 229), with an average of 0.69 (Table 4.1). The PIC values, a reflection of allele diversity and frequency among the cultivars, also varied from one locus to another. The PIC values derived from allelic diversity and frequency among the genotypes were not uniform for all the SSR loci. This indicated that all genotypes used in the present study were found to have enough diversity. The study found the highest PIC value (0.79) in RM 229, followed by 0.78 (RM 44), 0.77 (RM 60 & RM 225), 0.76 (RM 258), 0.74 (RM 31), 0.73 (RM 2), 0.72 (RM 241), 0.71 (RM 247), 0.66 (RM 208) and 0.59 (RM 237). Out of the twelve SSR markers used in this study, PIC values of the eleven SSR markers were greater than 0.5 (Table 4.1). Low PIC value of RM 201 SSR marker may be the result of closely related genotypes and high PIC values of RM 229 might be the result of diverse genotypes of cluster analysis indicating that the shared allele distance and cluster analysis were suitable to use the information derived from SSR markers. The study found that eleven SSR markers were considered to be the finest and highly informative. Therefore, it can be further used for molecular characterization and QTL mapping in rice.

4.3 Genetic Relationships

The rice genotypes is rich reservoir of valuable genes that plant breeders exploit it for crop improvement. The primers have the ability to differentiate different rice genotypes based on the differences in their genomic region and their number of alleles.

The Genetic Diversity (GD) values ranged from 0.22 (RM 201) to 0.81 (RM 229), with an average of 0.72 (Table4.1). The average genetic diversity value could be as a result of high rate of exchange of genetic materials among the rice genotypes studied mostly during their genetic improvement. Genetic diversity is key factor for germplasm conservation, characterization and breeding effects.

The Unweighted Paired Group Method Using Arithmetic Averages (UPGMA) clustering analysis was performed for genetic distance, and phylogenetic tree was constructed. UPGMA is one of the simplest clustering algorithms to generate distance-based phylogenetic dendrogram. The UPGMA cluster analysis of the 102 accessions, eight groups appeared to be related to their pedigrees (Figure 4.2). Group I includes only (2) accessions; Acc.1786 and Acc.1788 which possess modified glume (wings like structure). Group II comprised of (5) accessions which contains the local cultivars (e.g. Acc.0527 and Acc.2328). In group III, there were (19) accessions which included different local cultivars such as Acc.1546, Acc.1571 and Acc.1536. Including Myanmar popular varieties Paw San Hmwe (Acc. 1495) and Na Ma Tha Lay (Acc.1635), (17) accessions are included in the group IV. Group V consists of an accession of Paw San Hmwe (Acc. 2501) and (6) Myanmar local cultivars. In group VI, there were the biggest group mix with modern and local cultivars which contained (27) accessions. In group VII and VIII also contained (14) and (11) accessions with local and modern cultivars (Table 4.2). The UPGMA resulted in allelic richness of eight major clusters in which cluster VII is composed of a high number of accessions. Genotype from Yangon region fell into different clusters which suggests to use for the selections of parents for hybridization.

4.4 Population Structure Analysis

The model-based clustering method was performed using data of 12 SSR markers among 102 rice accessions. The relatively high value of K for 102 accessions was for K = 9 (Figure 4.3). At K = 9, the population structure analysis in the present study resulted in nine populations (Figure 4.4 & Table 4.3). The distribution of rice accession between genetic groups is quite different among accessions. The mixture is likely the result of breeding and domestication history, which have had large effects on the diversity structure. Human-mediated gene flow may play an important role within

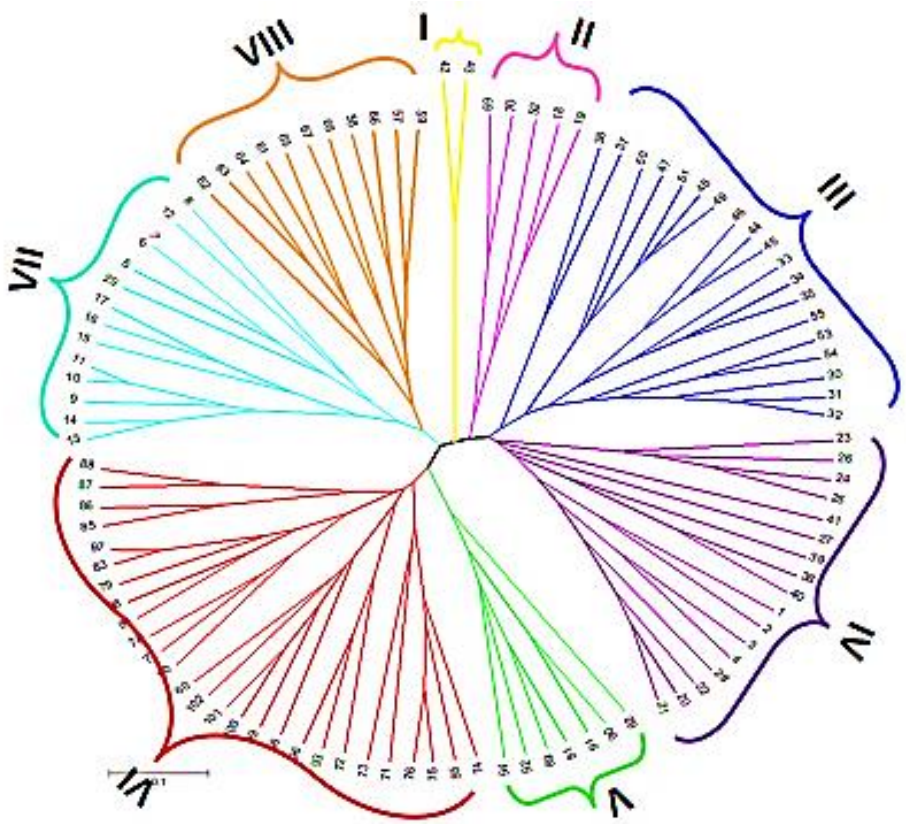
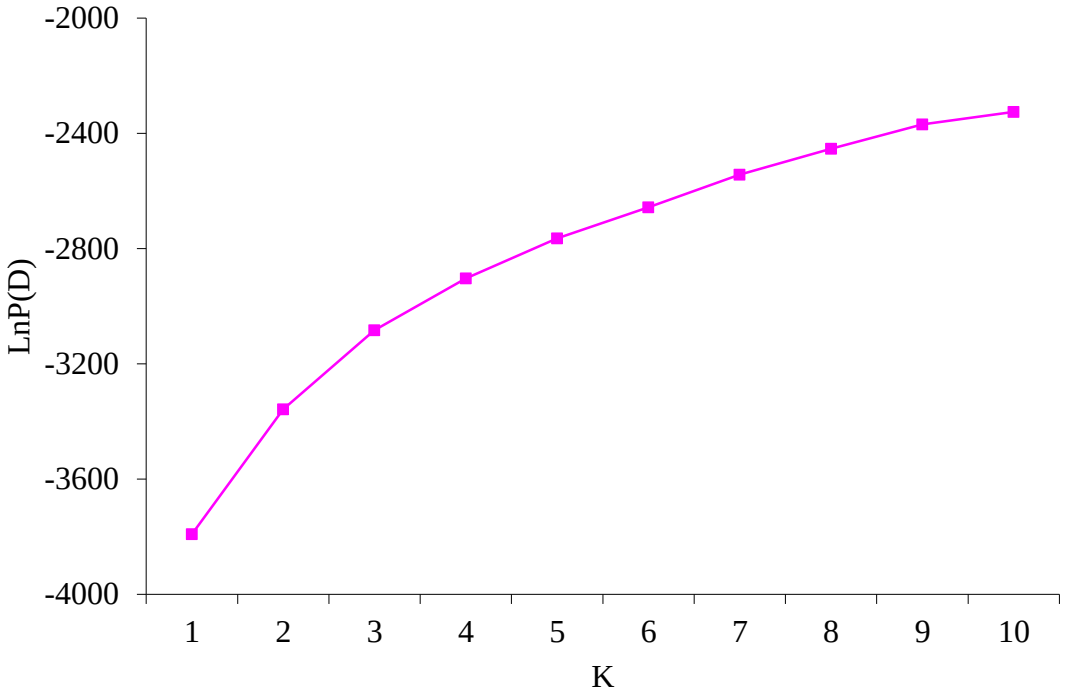


Figure 4.2 UPGMA dendrogram based on a genetic distance matrix among 102 rice accessions from Yangon Region in Myanmar

Table 4.2 Cluster groups of rice genotypes by UPGMA dendrogram

Cluster	Number of Accessions	Accession Number
I	2	Acc.1786, Acc.1788
II	5	Acc.2011, Acc.2328, Acc.1838, Acc.0529, Acc.0629
III	19	Acc.1601, Acc.1602, Acc.1832, Acc.1822, Acc.1835, Acc.1823, Acc.1827, Acc.1821, Acc.1808, Acc.1813, Acc.1546, Acc.1547, Acc.1571, Acc.1877, Acc.1840, Acc.1843, Acc.1505, Acc.1533, Acc.1536
IV	17	Acc.1359, Acc.1465, Acc.1373, Acc.1391, Acc.1635, Acc.1495, Acc.1629, Acc.1607, Acc.1631, Acc.0166, Acc.0169, Acc.0288, Acc.0310, Acc.1496, Acc.1338, Acc.0726, Acc.0746
V	7	Acc.2501, Acc.5941, Acc.5946, Acc.2496, Acc.5933, Acc.5951, Acc.6342
VI	27	Acc.2353, Acc.6352, Acc.2370, Acc.2425, Acc.2329, Acc.2331, Acc.2330, Acc.6263, Acc.6370, Acc.1883, Acc.9161, Acc.9162, Acc.9163, Acc.9790, Acc.1932, Acc.2488, Acc.2440, Acc.2441, Acc.2689, Acc.9150, Acc.2442, Acc.2506, Acc.6377, Acc.3727, Acc.5868, Acc.5926, Acc.5927
VII	14	Acc.0449, Acc.0465, Acc.0415, Acc.0417, Acc.0424, Acc.0466, Acc.0479, Acc.0510, Acc.1501, Acc.0323, Acc.0338, Acc.0355, Acc.0425, Acc.0367
VIII	11	Acc.1970, Acc.1978, Acc.1985, Acc.1955, Acc.1991, Acc.2003, Acc.2004, Acc.1894, Acc.2002, Acc.1884, Acc.1928

(a)



(b)

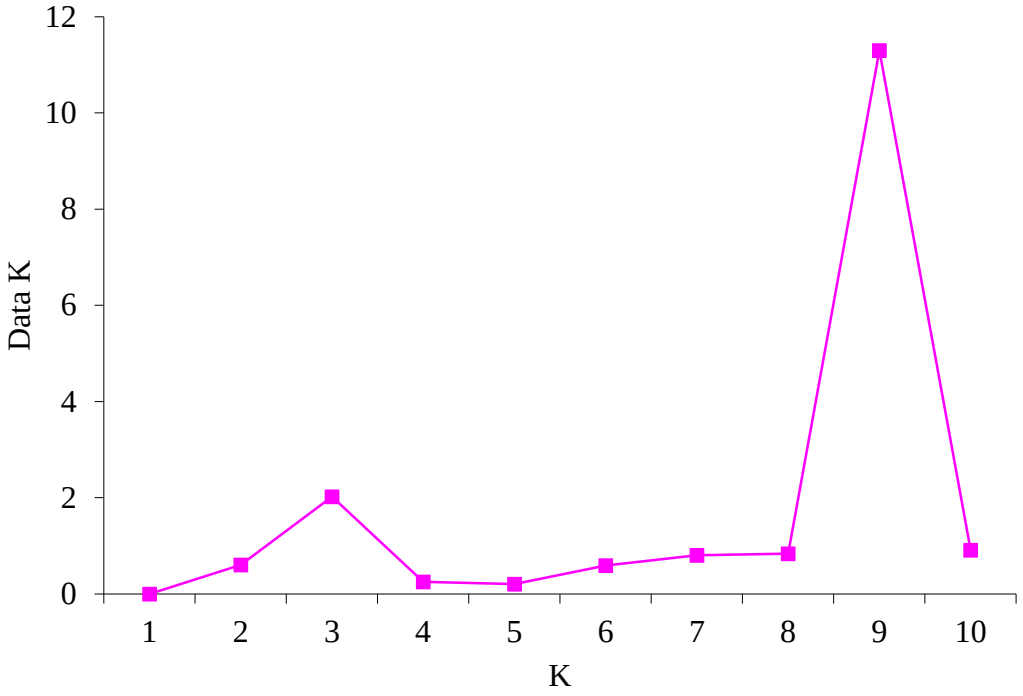


Figure 4.3 (a) The Bayesian log probability data [LnP (D)] by increasing K. (b) Magnitude of K as a function of K (Evanno, Regnaut, & Goudet, 2005)

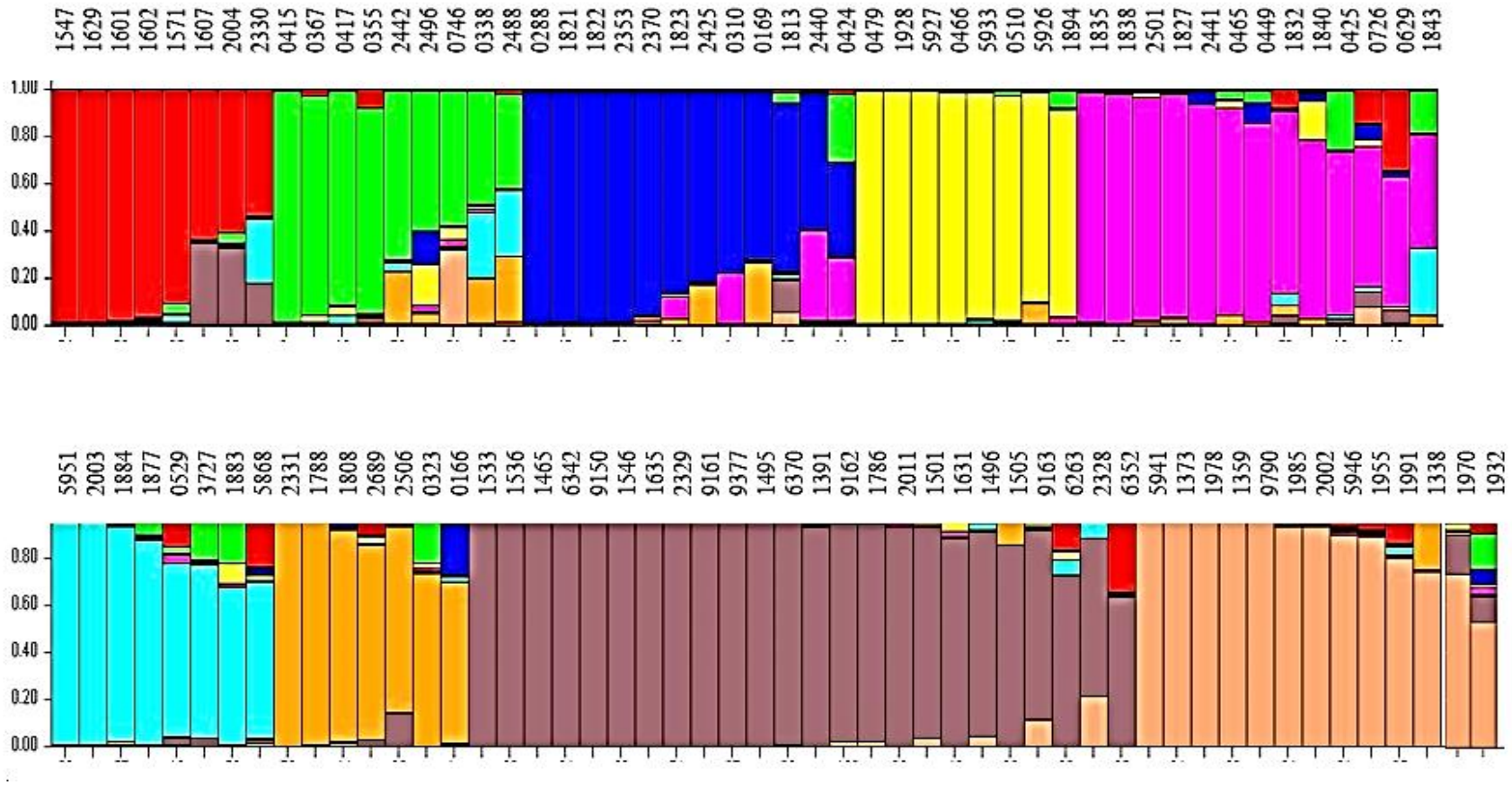


Figure 4.4 Model-based membership of 102 rice accessions using STRUCTURE. Colors represent model-based populations for 9 inferred cluster

Table 4.3 List of rice genotypes by population structure analysis

Population	Number of Accessions	Accession Number
A	8	Acc.1547, Acc.1629, Acc.1601, Acc.1602, Acc.1571, Acc.1607, Acc.2004, Acc.2330
B	9	Acc.0415, Acc.0367, Acc.0417, Acc.0355, Acc.2442, Acc.2496, Acc.0746, Acc.0338, Acc.2488
C	12	Acc.0288, Acc.1821, Acc.1822, Acc.2353, Acc.2370, Acc.1823, Acc.2425, Acc.0310, Acc.0169, Acc.1813, Acc.2440, Acc.0424
D	8	Acc.0479, Acc.1928, Acc.5927, Acc.0466, Acc.5933, Acc.0510, Acc.5926, Acc.1894
E	13	Acc.1835, Acc.1838, Acc.2501, Acc.1827, Acc.2441, Acc.0465, Acc.0449, Acc.1832, Acc.1840, Acc.0425, Acc.0726, Acc.0629, Acc.1843
F	8	Acc.5951, Acc.2003, Acc.1884, Acc.1877, Acc.0529, Acc.3727, Acc.1883, Acc.5868
G	7	Acc.2331, Acc.1788, Acc.1808, Acc.2689, Acc.2506, Acc.0323, Acc.0166
H	24	Acc.1533, Acc.1536, Acc.1465, Acc.6342, Acc.9150, Acc.1546, Acc.1635, Acc.2329, Acc.9161, Acc.9377, Acc.1495, Acc.6370, Acc.1391, Acc.9162, Acc.1786, Acc.2011, Acc.1501, Acc.1631, Acc.1496, Acc.1505, Acc.9163, Acc.6263, Acc.2328, Acc.6352
I	13	Acc.5941, Acc.1373, Acc.1978, Acc.1359, Acc.9790, Acc.1985,Acc.2002, Acc.5946, Acc.1955, Acc.1991, Acc.1338, Acc.1970, Acc.1932

a population due to breeding in rice for its self-fertilization nature (Zhao et al., 2009). In other words, in self-pollinated species, one would predict a greater partitioning of diversity among rather than within populations in the absence of human-mediated gene flow between populations by breeding (Kejun Liu et al., 2003).

In the present study, the average distances (except heterozygosity) between individuals of the same population were 0.3896 (population A), 0.4672 (population B), 0.3911 (population C), 0.3995 (population D), 0.4443 (population E), 0.6129 (population F), 0.4744 (population G), 0.5414 (population H), and 0.5533 (population I). The average genetic distances were used to evaluate the genetic diversity of different individuals (Table 4.4).

The information of genetic diversity and the population structure are useful in order to develop effective breeding strategies for broadening the genetic base of commercial varieties, to identify molecular tags, and for germplasm conservation. Molecular characterization of genotypes and detection of molecular tags will be useful in the Distinguish, Uniformity, and Stability (DUS) testing of plant varietal characterization, for development of essentially derived varieties.

Table 4.4 The average distances (except heterozygosity) between individuals of the same population

	Population A	Population B	Population C	Population D	Population E	Population F	Population G	Population H	Population I
AD	0.3896	0.4672	0.3911	0.3995	0.4443	0.6129	0.4744	0.5414	0.5533

AD = the average distances (except heterozygosity) between individuals of the same population

CHAPTER V
CONCLUSION

The 12 SSR primers revealed 91 alleles across the tested germplasm. High frequency of rare allele is important for maximizing the genetic variation in Yangon rice germplasm and to utilize them in varietal improvement. The presence of rare alleles indicate that these accessions are useful for plant breeders and geneticists as genetic source for rice breeding. Information available on these alleles present in different genotypes are useful for developing the mapping populations for genome analysis as well as in applied breeding programs.

The study found that the mean PIC value was 0.69 and PIC values of eleven SSR markers were greater than 0.5. Therefore, it can be further used for molecular characterization and QTL mapping in rice. The mean value of genetic diversity (0.72) depicted relative heterozygosity among the 102 rice genotypes. Results from this study revealed genetic diversity of rice genotypes in Yangon, which clearly indicated the importance of continued conservation and utilization of these germplasm in varietal development program for this region.

In the present study, genetic diversity was observed for only one lowland regions. If molecular characterization of local rice genotypes are studied in different regions of Myanmar, the genetic variation will be higher. Therefore, it should be continued to study for other local rice genotypes from different regions of Myanmar.

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APPENDICES

Appendix 1 Passport data and inferred populations of 102 rice genotypes

No.	Accession no.	Cultivar name	Township
1	0166	Lone Thwe Shwe War	Khayan
2	0169	C-4-63	Khayan
3	0288	Sein Kama Kyi	Hmawbi
4	0310	Thet Nu Saba Net	Kungyangon
5	0323	Shwe Pu	Khayan
6	0338	Nat Pyi Hmwe	Khayan
7	0355	Nyaung Aine	Kungyangon
8	0367	Nga Kywe	Hmawbi
9	0415	Nat Pyi Hmwe	Kungyangon
10	0417	Nat Pyi Hmwe	Khayan
11	0424	Pyi Daw Aye	Hmawbi
12	0425	Pyi Daw Aye	Hmawbi
13	0449	Kywe Chae Manaing	Khayan
14	0465	Bay Kyaung	Kungyangon
15	0466	C-64-1	Kungyangon
16	0479	Leik Kalay Nga Phyu	Kungyangon
17	0510	Shwe Kyi Dauk	Khayan
18	0529	Kauk Sann	Khayan
19	0629	Shwe Wa Hnan	Khayan
20	0726	Nga Kye Du Me	Hmawbi
21	0746	Yodaya	Hmawbi
22	1338	Mercury	Kungyangon
23	1359	A Pyo Gyi Paung Dan Shay	Hmawbi
24	1373	Emata Pin To	Hmawbi
25	1391	Emata Pin To	Hmawbi
26	1465	Nga Yaw Man	Hmawbi
27	1495	Paw San Hmwe	Kungyangon
28	1496	Hnan Su	Kungyangon
29	1501	Lone Gyi Kar	Hmawbi
30	1505	Nga Yun Wa Bo	Hmawbi
31	1533	Phut U	Hmawbi
32	1536	Ya Gyaw-2	Khayan
33	1546	Nga Cheik	Khayan
34	1547	Sein Shwe San	Hmawbi
35	1571	Nga Shink Thway	Kungyangon
36	1601	Leik Kalay Mee Don	Kungyangon
37	1602	Shwe War Mee Don	Kungyangon
38	1607	Shwe Palin	Khayan
39	1629	Kamar Kyi Shwe War	Khayan
40	1631	Hmaw Bi Nga Kywe	Khayan
41	1635	Na Ma Tha Lay	Kungyangon

Appendix 1 Passport data and inferred populations of 102 rice genotypes (Continued)

No.	Accession no.	Cultivar name	Township
42	1786	Thet Nu Saba Net Pyan	Kungyangon
43	1788	Nga Kywe Taung Pyan	Khayan
44	1808	C-68-18	Khayan
45	1813	Padan Nga Sein	Hmawbi
46	1821	Gwa Kama Kyi	Khayan
47	1822	Hnan Su	Kungyangon
48	1823	Hnan Su	Hmawbi
49	1827	Shwe Dinga	Kungyangon
50	1832	Ye Baw Sein	Kungyangon
51	1835	Shwe Chay Gyin	Hmawbi
52	1838	Tha Zin Tan	Hmawbi
53	1840	Nga Sein Kalar	Hmawbi
54	1843	Ya Ma Gyi	Hmawbi
55	1877	Let Ywe Zin	Hmawbi
56	1883	Nga Yar Po	Khayan
57	1884	Go Kaung	Hmawbi
58	1894	Moe Thay	Khayan
59	1928	Kauk Hnyin	Kungyangon
60	1932	Kywet Thwa	Khayan
61	1955	Nga Sein Thee Dat	Khayan
62	1970	Ye Laik Mee Don	Kungyangon
63	1978	Thu Ka De Thee Dat	Thongwa
64	1985	Nga Sein Gaung Me	Hmawbi
65	1991	Kalai Nga Sein	Khayan
66	2002	Kauk Hnyin Khun Ni	Khayan
67	2003	Shwe Palin	Hmawbi
68	2004	Hnget Pyaw Nyunt	Khayan
69	2011	Shwe Wa Hmwe	Khayan
70	2328	Mee Kauk	Kungyangon
71	2329	Pin Do Sein	Kungyangon
72	2330	Kauk Yin Shwe War	Khayan
73	2331	Kamar Kyi	Khayan
74	2353	Sit Pwa	Hmawbi
75	2370	Shwe Wa Mee Don	Kungyangon
76	2425	Kywe Pu	Hmawbi
77	2440	Pan Thit Sa	Hmawbi
78	2441	Ye Kyaw	Khayan
79	2442	Hmaw Bi Nga Sein	Hmawbi
80	2488	Shwe Palin	Khayan

Appendix 1 Passport data and inferred populations of 102 rice genotypes (Continued)

No.	Accession no.	Cultivar name	Township
81	2496	Pathein Nyunt	Kungyangon
82	2501	Paw San Hmwe	Khayan
83	2506	Ma Sein Aye	Hmawbi
84	2689	Shwe Wa Gyi	Kungyangon
85	3727	Taung Paw Khin Sein	Hmawbi
86	5868	B-541-B-KN-58-5-3	Hmawbi
87	5926	Khao Note Tit Own	Hmawbi
88	5927	Khao Kwe Lan	Hmawbi
89	5933	Pawa Nyo	Hmawbi
90	5941	Ye Manaing Kauk Kyi	Hmawbi
91	5946	Shwe War Gyi	Hmawbi
92	5951	Buzayet	Hmawbi
93	6263	Shwe Sayar	Hmawbi
94	6342	Taing Thi Lauk	Hmawbi
95	6352	Kauk Hnyin Gyi	Hmawbi
96	6370	Moe Ma Kha Gyi	Hmawbi
97	6377	Byat Nga Kywe	Hmawbi
98	9150	Kan Yoe Tan-1	
99	9161	Shwe Bo (1)	
100	9162	Hmaw Bi (3)	
101	9163	Kan Yoe Tane (1)	
102	9790	Shwe Bo -1	

Appendix 2 Procedure for DNA extraction method (Isoproponol precipitation method)

Step 1 : A few pieces of rice young leave (3-4cm) is collected and cut into small pieces (5mm) using scissors and add into 1.5ml microtube.

Step 2 : Added 200 µl of T10E1 buffer (10mMTris HCl: 1mM EDTA) at pH 8 into micro tube and grind pieces of leave using homogenizer.

Step 3 : The 250 µl of T10E1 buffer (10mMTris HCl: 1mM EDTA) at pH 8 is again added.

Step 4 : After lasting 1 hour, it is centrifuged the tubes at 10˚C and 15,000 rpm for 10 minutes.

Step 5 : The 400 µl of supernatant is transferred into new microtube.

Step 6 : The 300 µl of isopropanol is added into the new tube containing supernatant and then shake the tube to appear reaction.

Step 7 : It is centrifuged the tubes at 10˚C and 15,000 rpm for 10 minutes.

Step 8 : Discard the supernatant.

Step 9 : The DNA pallet is washed with 1000 µl of chilled 70% ethanol.

Step 10 : It is centrifuged the tubes at 10˚C and 15,000 rpm for 1 minutes.

Step 11 : Collect the DNA pallet by discarding the chilled 70% ethanol.

Step 12 : It is dried the DNA pallet at room temperature.

Step 13 : Dissolve the DNA pallet in the 500 µl of T10E0.1 buffer.

Step 14 : Shake the tube by hand to dissolve the DNA pallet in buffer and stored at -20ºC for future use.