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Comparative Evaluation of Rice SSR Markers on Different *Oryza* Species

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Abstract

The growing number of rice microsatellite markers permit a comprehensive comparison of allelic variation among the markers developed using different methods, with diverse repeat motifs and at variable genomic regions. Under this study, comparison between a set of 67 microsatellite markers representing the whole (twelve) rice chromosomes was done over worldwide collections of nine species of the genus *Oryza*. These SSR markers were evaluated for the genetic parameters such as; number of alleles amplified per primers, observed heterozygosity, gene diversity, rare allelic frequency and Polymorphic Information Content (PIC) values. Among the microsatellite markers that were assessed in the present study, highest overall degree of genetic diversity was recorded on a dinucleotide repeat motif containing markers. Therefore, employing such very informative markers for future molecular characterization and diversity study of *Oryza* species is advisable. The unique alleles generated from those polymorphic markers could also have significant role on the efforts of conservation, population genetics study and identification of both wild and cultivated *Oryza* species.

Keywords

Genetic diversity, Microsatellite markers, *Oryza*, Simple sequence repeats (SSR)

Abbreviations

F: Fixation Index; F_{is}: Wrights Fixation Index; F_{it}: Total Inbreeding Coefficient; F_{st}: Genetic Differentiation; He: Expected Heterozygosity; Ho: Observed Heterozygosity; Na: Actual Number of Alleles; Ne: Effective Number of Alleles; PIC: Polymorphic Information Content; PCR: Polymerase Chain Reaction; RM: Rice Marker; SSR: Simple Sequence Repeats

Introduction

The Asian cultivated rice (*Oryza sativa* L.) which feeds more than one third of the world's population is categorized under the genus *Oryza* [1]. A variety of local landraces and cultivars of rice are found in the *indica* and *japonica* subspecies [2,3]. Other than these two huge reservoirs of rice germplasm, over 20 wild *Oryza* species constitute an exceptionally valuable gene pool for rice improvement [4]. Thus, knowledge on the level of genetic diversities and species relationships in the genus *Oryza* is essential for efficient strategies targeting collection, conservation and introgression of useful genes to cultivated rice [5]. In rice breeding, hybridization between parental lines with a defined genetic distance and subsequent selection is a common approach [1]. These days, information on the extent of such genetic relationships between genotypes for an effective breeding program is generated from diverse molecular marker-based techniques [6].

Molecular markers are powerful tools in the assessment of genetic variation and elucidation of genetic relationships within and among plant species [7-9]. Likewise, molecular markers have been used in the genus *Oryza* to identify accessions [10], determine genetic structure and pattern of diversity [11], and optimize assembly of core collections [12]. Earlier molecular marker types such as RAPD, ISSR and AFLP have been used very frequently for fingerprinting and characterization of varieties and germplasm accessions [13]. However, they can be utilized without prior genomic information on the target crop and thus considered as markers of choice [6]. Since 2000, the locus specific Simple Sequence Repeat (SSR) markers got preferential application in cultivar identification of many crops such as, rice [14].

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SSRs are highly frequent 1 to 6 bp repeated motifs distributed throughout the nuclear [15], chloroplast [16,17] and mitochondrial [18] genomes. Unique simple sequence repeat (SSR) profiles in rice cultivars can be generated by using few primers covering all of the 12 chromosomes [19]. However, a total of 18,828 Class 1 di-, tri- and tetra-nucleotide SSRs, representing 47 distinctive motif families, were identified and annotated on the rice genome [1]. The published high-density linkage map indicated abundance of microsatellite markers (with an average of 51 hypervariable SSRs per Mb) with the highest density of markers at chromosome 3 (55.8 SSRMb-1) and at chromosome 4 (41.0 SSRMb-1) [20].

Such neutral and co-dominant SSR markers have merits like fast assay, technical simplicity, high polymorphism and stability [21]. Besides, the reproducible and cross-species transferable feature of SSR markers make them as valuable tools for genetic diversity study [9]. Among the most widely used DNA marker types, SSR markers are widely applied in gene mapping [22,23], establishment of genetic relationships [15,24], construction of fingerprints [23,25], genetic purity test [26], molecular evolution studies [21], varietal identification and heterosis utilization [9]. For instance, clustering data generated from SSR makers were used for identifying a hybrid with highest heterosis from the intercrossing between different and distantly related *Oryza* species [1].

Variations on the molecular basis of polymorphism and distribution across the genome allow the different SSR marker types with different views for a given population structure [6]. The high degree of length polymorphism or variable allelic size by different SSR markers may also be attributed to chromosomal rearrangements during the genome's evolution, the different number of repeats in the SSR regions or replication slippage mechanism [21]. Differences in the level of SSR polymorphism may also be associated with gene conservation, source of the SSRs (genomic and EST-SSR) and/or nature of the SSRs' nucleotide repeat unit (such as di-, tri- and tetra) [6]. Besides, [27] reported markers having perfect dinucleotide (GA and CT) repeat motifs as markers with high level of variation among rice genotypes.

Many studies over diverse types of molecular markers reported SSRs as markers with highly significant allelic variation [28]. Allelic variation for tested SSR loci defines polymorphism among varieties or the polymorphism information content (PIC) value [1]. Hence, utility of SSR markers for assessment of genetic diversity, population structure study and crop improvement depend on quality of PIC they provide [6]. Indirectly, prior choice of markers with highly informative and polymorphic rice SSR markers-based study is too critical to conserve rice genotypes, reveal gene pool of rice landraces and unlock valuable genes for breeding purposes [29]. Thus, the present study was conducted to compare the widely used 67 selected rice SSR markers (RMs) for the assessment of genetic variability and population structure among 426 samples of 9 *Oryza* species. SSR markers evaluation over worldwide *Oryza* species collections for statistical and genetic parameters such as number of alleles amplified per primers, gene diversity, heterozygosity and PIC value identified markers which are more informative and promising for future genetic investigation on both wild and cultivated rice types.

Materials and Methods

Plant material

A total of 426 rice samples, hereafter referred as "accessions" comprising 35 accessions of cultivated rice germplasms from Yunnan University gene bank (Table 1), 31 accessions of 7 AA and CC genome wild *Oryza* species from IRRI (Table 2) and 360 collections from 12 Ethiopian populations of the African wild rice (*O. longistaminata*) (Table 3) were included in this study.

Table 1: 35 varieties of cultivated rice.

Sample ID	Species/Subspecies	Genome type	Accession code	Origin
P01	*Japonica*	AA	Nippobrane	Yunnan university gene bank
P02	*Japonica*	AA	zhonghua11	Yunnan university gene bank
P03	*Japonica*	AA	Kitaake	Yunnan university gene bank
P04	*Indica*	AA	RD23	Yunnan university gene bank
P05	*Indica*	AA	minghui63	Yunnan university gene bank
P06	*Japonica*	AA	Koshihikari	Yunnan university gene bank
P07	*Japonica*	AA	yundao2	Yunnan university gene bank
P08	*Japonica*	AA	chujing27	Yunnan university gene bank
P09	*Indica*	AA	9311	Yunnan university gene bank
P10	*Indica*	AA	luyin46	Yunnan university gene bank
P11	*Indica*	AA	Huanghuazhan	Yunnan university gene bank
P12	*Japonica*	AA	IRAT104	Yunnan university gene bank
P13	*Indica*	AA	IR64	Yunnan university gene bank
P14	*Indica*	AA	guichao2	Yunnan university gene bank
P15	*Japonica*	AA	Xueheaizao	Yunnan university gene bank
P16	*Japonica*	AA	Hejiang19	Yunnan university gene bank
P17	*Japonica*	AA	Pokkali	Yunnan university gene bank
P18	*Indica*	AA	IR29	Yunnan university gene bank
P19	*Indica*	AA	Mengwanggu	Yunnan university gene bank

P20	*Indica*	AA	taiwanludao1	Yunnan university gene bank
P21	*Japonica*	AA	chujing28	Yunnan university gene bank
P22	*Japonica*	AA	dianjingyou5	Yunnan university gene bank
P23	*Indica*	AA	wendao12	Yunnan university gene bank
P24	*Japonica*	AA	hexi41	Yunnan university gene bank
P25	*Indica*	AA	yunhui290	Yunnan university gene bank
P26	*Indica*	AA	banna21	Yunnan university gene bank
P27	*Japonica*	AA	Akitakomati	Yunnan university gene bank
P28	*Japonica*	AA	Akihikari	Yunnan university gene bank
P29	*Indica*	AA	dianrui449	Yunnan university gene bank
P30	*Indica*	AA	diantun502	Yunnan university gene bank
P31	*Japonica*	AA	PR23	Yunnan university gene bank
P37	*Japonica*	AA	yunjing37	Yunnan university gene bank
P41	*Indica*	AA	mengwangmaxiangu	Yunnan university gene bank
P42	*Indica*	AA	Mengwangdabaigu	Yunnan university gene bank
P43	*Indica*	AA	mengwangxiaobaigu	Yunnan university gene bank

Table 2: 35 accessions representing 10 wild *Oryza* species and 1 weedy rice.

Sample ID	Species	Genome type	Accession No	Origin
YSD16	*O. barthii*	AA	Acc.100921	
WR23	*O. barthii*	AA	Acc.104061	Niger
WR26	*O. barthii*	AA	Acc.104284	Mali
YSD88	*O. glumaepatula*	AA	Acc.100894(W)	Cuba
YSD90	*O. glumaepatula*	AA	Acc.103812(W)	Brazil
YSD92	*O. glumaepatula*	AA	Acc.105661	Brazil
YSD95	*O. glumaepatula*	AA	Acc.105662	Brazil
WR88	*O. glumaepatula*	AA	Acc.100894(D)	Cuba
WR90	*O. glumaepatula*	AA	Acc.103812(D)	Brazil
YSD64	*O. nivara*	AA	Acc.80611	India
YSD66	*O. nivara*	AA	Acc.80677	India
WR60	*O. nivara*	AA	Acc.80432	India
WR65	*O. nivara*	AA	Acc.80625	India
WR77	*O. nivara*	AA	Acc.80696	India
WR78	*O. nivara*	AA	Acc.81855	India
YSD42	*O. rufipogon*	AA	Acc.82040	Thailand
YSD46	*O. rufipogon*	AA	Acc.106133(W)	India
YSD52	*O. rufipogon*	AA	PCR9607(D)	Thailand
WR40	*O. rufipogon*	AA	Acc.81994	Papua New Guinea
WR46	*O. rufipogon*	AA	Acc.106133	India
WR48	*O. rufipogon*	AA	Acc.106340	Myanmar
WR55	*O. rufipogon* open pollinated	AA	Acc.105832(W)	Bgo2,1998 gkanlaus
WR57	*O. rufipogon* open pollinated	AA	Acc.106138(W)	Bgo2,2001 gkanlaus
YSD55	*O. rufipogon* open pollinated	AA	Acc.105832(D)	Bgo2,1998 gkanlaus
YSD57	*O. rufipogon* open pollinated	AA	Acc.106138(D)	Bgo2,2001 gkanlaus
Lg	*O. longistaminata*	AA		Niger
YSD01	*O. officinalis*	CC	8712	Sukhothai
YSD05	*O. rhizomatis*	CC	W95018(W)	IRRI
WR05	*O. rhizomatis*	CC	W95018(D)	IRRI
WR03	*O. eichingeri*	CC	Acc.105181(W)	Uganda
YSD03	*O. eichingeri*	CC	Acc.105181(D)	Uganda
YSD08	*O. latifolia*	CCDD	Acc.100169	Costa Rice
WR09	*O. australiensis*	EE	Acc.101410(W)	Australia
YSD09	*O. australiensis*	EE	Acc.101410(D)	Australia
YSD58	Japonica weedy type		96036	Korea

Genomic DNA extraction and polymerase chain reaction (PCR)

Total genomic DNA was extracted from fresh leaves by using CTAB protocol as described by [30]. Quality for the extracted DNA was determined by electrophoresis in a 1% agarose gel and quantification was accomplished using a spectrophotometer. For SSR analyses, the extracted DNA samples were diluted to 20 ng/µl using TE (Tris-EDTA) and stored at -20 °C.

A total of Sixty-seven nuclear SSR markers covering the 12 rice chromosomes were used in this study (Table 4). Those polymorphic SSR markers were selected for amplification according to the reports from [27,31,32]. Polymerase chain reaction (PCR) was done by using a 10 µl reaction mixture in a 96-well plate. Each reaction mixture contained 4 µl

Table 3: Ecology of the Ethiopian *O. longistaminata* populations.

Group code	Site name	Coordinate 1	Coordinate 2	Elevation	Ecology type
Gambella1	Gambella Agricultural Research Institute	36P0673586	UTM0912603	431M	In a small stream with no rice cultivation history
Gambella2	Kera	36P0673826	UTM0912320	438M	A wetland which gets dry in a dry season and without a rice cultivation practice
Gambella3	Elai	36P0674462	UTM0911700	442M	Baro river provides water and had rice cultivation trial in 2011
Gambella4	Echewi	36P0676012	UTM0909521	443M	A swampy area without any rice production practice
Gambella5	Aleworo	36N0667699	UTM0872871	460M	An isolated area and free from any rice cultivation
Gambella6	Catholic Church	36N0667515	UTM0872896	457M	A semi-wetland area without rice production history
Amhara1	Tana Hotel	37P0325270	UTM1283260	1,800M	Tana lake is the source of water and it is an isolated area with no rice production practice
Amhara2	Tewa Zakena	37P0359496	UTM1320023	1,801M	A stream water near the road is the source and there is a rice farm at 100 Meter away from the collection site
Amhara3	Abwa Kokite School	37P0359239	UTM1324519	1,801M	Many rice varieties by the Woreta Agricultural Institute has been grown at 50 M distance
Amhara4	Gami Gote	37P0316212	UTM1294335	1,790M	Had a trial to cultivate rice in 2011 and 2012 but the overflow of water from Tana lake makes it less likely
Amhara5	Wenjeta Kebelle	37P0314305	UTM1291528	1,792M	A rice farm from 2009-2013. Though, it is now abandoned
Amhara6	Fessesa	37P0313974	UTM1292507	1,798M	A rice farm up to 2014

Table 4: List of primers used in the PCR with their 5'-3' sequence, repeat motif's, chromosomal location and annealing temperature.

Primer	5'-3' sequence of FWD/REV primer	SSR repeat motif	Chromosomal location	Annealing temperature
RM5	FWD: TGCAACTTCTAGCTGCTCGA	(GA)14	1	
	REV: GCATCCGATCTTGATGGG			55
RM6	FWD: TGCAACTTCTAGCTGCTCGA	(AG)16	2	
	REV: TCGTCTACTGTTGGCTGCAC			55
RM11	FWD: TCTCCTCTTCCCCCGATC	(GA)17	7	
	REV: ATAGCGGGCGAGGCTTAG			55
RM14	FWD: CCGAGGAGAGGAGTTCGAC	(GA)17	1	
	REV: GTGCCAATTTCCTCGAAAAA			55
RM17	FWD: TGCCCTGTTATTTTCTTCTCTC	(GA)21	12	
	REV: GGTGATCCTTTCCCATTTCA			55
RM19	FWD: CAAAAACAGAGCAGATGAC	(ATC)10	12	
	REV: CTCAAGATGGACGCCAAGA			55
RM21	FWD: ACAGTATTCCGTAGGCACGG	(GA)21	11	
	REV: GCTCCATGAGGGTGGTAGAG			55
RM22	FWD: GGTTTGGGAGCCCATAATCT	(GA)22	3	
	REV: CTGGGCTTCTTTCACTCGTC			55
RM23	FWD: CATTGGAGTGGAGGCTGG	(GA)15	1	
	REV: GTCAGGCTTCTGCCATTCTC			55

RM24	FWD: GAAGTGTGATCACTGTAACC	(GA)29	1	
	REV: TACAGTGGACGGCGAAGTCG			55
RM25	FWD: GGAAAGAATGATCCTTTTCATGG	(GA)18	8	
	REV: CTACCATCAAAACCAATGTTC			55
RM26	FWD: GAGTCGACGAGCGGCAGA	(GA)15	5	
	REV: CTGCGAGCGACGGTAACA			55
RM29	FWD: CAGGGACCCACCTGTCATAC	(GA)7-18(GA)5(AG)4	2	
	REV: AACGTTGGTCATATCGGTGG			55
RM30	FWD: GGTTAGGCATCGTCACGG	(AG)9A(AGA)12	6	
	REV: TCACCTCACCACACGACACG			55
RM31	FWD: GATCACGATCCACTGGAGCT	(GA)15	5	
	REV: AAGTCCATTACTCTCCTCCC			55
RM42	FWD: ATCCTACCGCTGACCATGAG	(AG)5(GA)(AG)2T(GA)A(AG)5	8	
	REV: TTTGGTCTACGTGGCGTACA			55
RM44	FWD: ACGGGCAATCCGAACAACC	(GA)16	8	
	REV: TCGGGAAAACCTACCCTACC			55
RM49	FWD: TTCGGAAGTTGGTTACTGATCA	(GA)27	3	
	REV: TTGGAGCGGATTCGGAGG			55
RM50	FWD: ACTGTACCGGTCGAAGACG	(CTAT)4(CT)15	6	
	REV: AAATTCCACGTCAGCCTCC			55
RM55	FWD: CCGTCGCCGTAGTAGAGAAG	(GA)17	3	
	REV: TCCCGGTTATTTTAAGGCG			55
RM60	FWD: AGTCCCATGTTCCACTTCCG	(AATT)5AATCT(AATT)	3	
	REV: ATGGCTACTGCCTGTACTAC			55
RM80	FWD: TTGAAGGCGCTGAAGGAG	(TCT)25	8	
	REV: CATCAACCTCGTCTTCACCG			55
RM84	FWD: TAAGGGTCCATCCACAAGATG	(TCT)10	1	
	REV: TTGCAAATGCAGCTAGAGTAC			55
RM105	FWD: GTCGTCGACCCATCGGAGCCAC	(CCT)6	10	
	REV: TGGTCGAGGTGGGGATCGGGTC			55
RM110	FWD: TCGAAGCCATCCACCAACGAAG	(GA)15	2	
	REV: TCCGTACGCCGACGAGGTCGAG			55
RM118	FWD: CCAATCGGAGCCACCGGAGAGC	(GA)8	7	
	REV: CACATCCTCCAGCGACGCCGAG			67
RM125	FWD: ATCAGCAGCCATGGCAGCGACC	(GCT)8	7	55
	REV: AGGGGATCATGTGCCGAAGGCC			
RM126	FWD: CGCGTCCGCGATAAACACAGGG	(GA)7	8	
	REV: TCGCACAGGTGAGGCCATGTCG			55
RM129	FWD: TCTCTCCGGAGCCAAGGCGAGG			
	REV: CGAGCCACGACGCGATGTACCC	(CGG)8	1	55
RM133	FWD: TTGGATTGTTTTGCTGGCTCGC	(CT)8	6	
	REV: GGAACACGGGGTCGGAAGCGAC			60
RM134	FWD: ACAAGGCCGCGAGAGGATTCCG			
	REV: GCTCTCCGGTGGCTCCGATTGG	(CCA)7	7	55
RM136	FWD: GAGAGCTCAGCTGCTGCCTCTAGC	(AGG)7	6	
	REV: GAGGAGCGCCACGGTGTACGCC			55
RM144	FWD: TGCCCTGGCGCAAATTTGATCC	(ATT)11	11	
	REV: GCTAGAGGAGATCAGATGGTAGTGCATG			57
RM152	FWD: GAAACCACCACACCTCACCG	(GGC)10	8	
	REV: CCGTAGACCTTCTTGAAGTAG			55
RM159	FWD: GGGGCACTGGCAAGGGTGAAGG	(GA)19	8	
	REV: GCTTGTGCTTCTCTCTCTCTCTCTCTCTC			55
RM161	FWD: TGCAGATGAGAAGCGGCGCCTC	(AG)20	5	
	REV: TGTGTCATCAGACGGCGCTCCG			57
RM162	FWD: GCCAGCAAAACCAGGGATCCGG	(AC)20	6	
	REV: CAAGGTCTTGTGCGGCTTGCGG			61

RM171	FWD: AACGCGAGGACACGTACTTAC	(GATG)5	10	
	REV: ACGAGATACGTACGCCTTTG			55
RM184	FWD: ATCCCATTCGCCAAAACCGGCC	(CA)7	10	55
	REV: TGACACTTGGAGAGCGGTGTGG			
RM205	FWD: CTGGTTCTGTATGGGAGCAG	(GA)25	9	
	REV: CTGGCCCTTCACGTTTCAGTG			55
RM207	FWD: CCATTCGTGAGAAGATCTGA	(CT)25	2	
	REV: CACCTCATCCTCGTAACGCC			55
RM209	FWD: ATATGAGTTGCTGTCGTGCG	(CT)18	11	
	REV: CAACTTGCATCCTCCCCTCC			55
RM212	FWD: CCACTTTCAGCTACTACCAG	(GA)24	1	
	REV: CACCCATTTGTCTCTCATTATG			55
RM213	FWD: ATCTGTTTGCAGGGGACAAG	(CT)17	2	55
	REV: AGGTCTAGACGATGTCGTGA			
RM215	FWD: CAAAATGGAGCAGCAAGAGC	(GA)16	9	
	REV: TGAGCACCTCCTTCTCTGTAG			55
RM219	FWD: CGTCGGATGATGTAAAGCCT	(GA)17	9	
	REV: CATATCGGCATTCGCCTG			55
RM220	FWD: GGAAGGTAACTGTTTCCAAC	(CT)17	1	
	REV: GAAATGCTTCCCACATGTCT			55
RM224	FWD: ATCGATCGATCTTCACGAGG	(AAG)8(AG)13G(AG)	11	
	REV: TGCTATAAAAGGCATTCGGG			55
RM225	FWD: TGCCCATATGGTCTGGATG	(CT)18	6	
	REV: GAAAGTGGATCAGGAAGGC			55
RM333	FWD: GTACGACTACGAGTGTCACCAA	(TAT)19(CTT)19	10	55
	REV: GTCTTCGCGATCACTCGC			
RM337	FWD: GTAGGAAAGGAAGGGCAGAG	CTT64 8 (CTT)4-19-(CTT)8	8	55
	REV: CGATAGATAGCTAGATGTGGCC			
RM153	FWD: GCCTCGAGCATCATCATCAG	(GAA)9	5	55
	REV: ATCAACCTGCACTTGCCTGG			
RM154	FWD: ACCCTCTCCGCCTCGCCTCCTC	(GA)21	2	61
	REV: CTCCTCCTCCTGCGACCGCTCC			
RM164	FWD: TCTTGCCCGTCACTGCAGATATCC	(GT)16TT(GT)4	6	55
	REV: GCAGCCCTAATGCTACAATTCTTC			
RM180	FWD: CTACATCGGCTTAGGTGTAGCAACACG	(ATT)10	7	55
	REV: ACTTGCTCTACTTGTGGTGAGGGACTG			
RM206	FWD: TAGTTTAACCAAGACTCTC	(GA)19	2	55
	REV: GGTTGAACCCAAATCTGCA			
RM307	FWD: GTACTACCGACCTACCGTTCAC	(AT)14(GT)21	4	55
	REV: CTGCTATGCATGAACTGCTC			
RM322	FWD: CAAGCGAAAATCCCAGCAG	(CAT)7	2	55
	REV: GATGAAACTGGCATTGCCTG			
RM1	FWD: GCGAAAACACAATGCAAAAA	(GA)26	1	55
	REV: GCGTTGGTTGGACCTGAC			
RM211	FWD: CCGATCTCATCAACCAACTG	(TC)3A(TC)18	2	55
	REV: CTTCACGAGGATCTCAAAGG			
RM251	FWD: GAATGGCAATGGCGCTAG	(CT)29	3	55
	REV: ATGCGGTTCAAGATTCGATC			
RM263	FWD: CCCAGGCTAGCTCATGAACC	(CT)34	2	55
	REV: GCTACGTTTGAGCTACCACG			
RM167	FWD: GATCCAGCGTGAGGAACACGT	GGAA(GA)16GGGG	11	55
	REV: AGTCCGACCACAAGGTGCGTTGTC			
RM216	FWD: TTCCCCAATGGAACAGTGAC	(CT)18	10	55
	REV: AGGGTCTACCACCCGATCTC			
RM475	FWD: CCTCACGATTTTCCTCCAAC	(TATC)8		
	REV: ACGGTGGGATTAGACTGTGC		2	55

RM6344	FWD: ACACGCCATGGATGATGAC	(GAA)8	7	55
	REV: TGGCATCATCACTTCCTCAC			
RM335	FWD: GTACACACCCACATCGAGAAG	(CTT)25	4	55
	REV: GCTCTATGCGAGTATCCATGG			

Table 5: Genetic diversity indices revealed by 67 SSR markers on 430 wild, weedy and cultivated rice accessions.

Marker type	Major Allele Frequency	No of rare alleles	Allele No	PIC	Ne	Ho	He	F_{is}	F_{it}	F_{st}	Nm
RM125	0.67	1	5	0.47	1.48	0.18	0.23	0.23	0.75	0.67	0.12
RM1	0.34	1	7	0.72	2.3	0.22	0.46	0.52	0.67	0.31	0.55
RM263	0.45	1	5	0.69	1.98	0.43	0.43	0.02	0.37	0.36	0.45
RM337	0.53	0	4	0.46	1.61	0.02	0.24	0.9	0.97	0.65	0.14
RM211	0.51	2	5	0.54	1.38	0.04	0.19	0.8	0.88	0.41	0.36
RM154	0.4	0	5	0.62	1.68	0.13	0.28	0.53	0.82	0.62	0.15
RM180	0.56	0	5	0.54	1.06	0.04	0.23	0.84	0.96	0.73	0.09
RM167	0.81	5	8	0.28	1.46	0	0.23	1	1	0.58	0.18
RM164	0.5	2	7	0.58	1.52	0.01	0.25	0.97	0.99	0.68	0.12
RM251	0.86	3	8	0.24	2	0.1	0.38	0.74	0.86	0.48	0.27
RM110	0.56	2	6	0.41	1.92	0.37	0.44	0.15	0.5	0.41	0.36
RM322	0.87	3	6	0.22	1.83	0.35	0.38	0.09	0.45	0.4	0.38
RM206	0.6	6	13	0.57	1.39	0.03	0.3	0.89	0.96	0.65	0.13
RM84	0.54	3	11	0.63	2.14	0.31	0.47	0.33	0.59	0.39	0.39
RM216	0.79	2	7	0.33	1.39	0.04	0.21	0.82	0.95	0.7	0.11
RM6344	0.52	5	9	0.55	1.67	0.12	0.3	0.59	0.85	0.62	0.15
RM307	0.86	1	7	0.24	1.7	0.08	0.27	0.69	0.89	0.64	0.14
RM333	0.52	1	9	0.49	1.96	0.09	0.33	0.74	0.89	0.58	0.18
RM153	0.9	1	6	0.18	1.55	0.07	0.25	0.71	0.9	0.65	0.13
RM5	0.74	3	9	0.42	1.66	0.19	0.33	0.43	0.76	0.58	0.18
RM475	0.36	2	5	0.65	0.88	0.05	0.2	0.77	0.95	0.78	0.07
RM6	0.37	1	8	0.61	1.64	0.05	0.31	0.83	0.93	0.58	0.18
RM11	0.76	4	6	0.38	1.65	0.04	0.3	0.87	0.93	0.49	0.26
RM17	0.54	0	4	0.53	1.49	0.2	0.29	0.31	0.68	0.54	0.21
RM19	0.92	1	4	0.14	1.13	0.01	0.12	0.89	0.98	0.83	0.05
RM21	0.47	5	7	0.64	1.47	0.07	0.26	0.73	0.88	0.54	0.21
RM23	0.44	4	8	0.60	1.45	0.03	0.24	0.87	0.96	0.67	0.12
RM24	0.67	1	4	0.40	1.65	0.03	0.31	0.91	0.95	0.51	0.24
RM25	0.41	1	6	0.60	1.51	0	0.29	1	1	0.67	0.13
RM42	0.46	3	5	0.53	1.26	0.01	0.18	0.92	0.97	0.57	0.19
RM31	0.5	3	7	0.62	1.62	0	0.28	1	1	0.57	0.19
RM30	0.74	2	7	0.41	1.58	0.01	0.27	0.97	0.99	0.62	0.15
RM29	0.91	2	5	0.15	1.34	0	0.19	1	1	0.73	0.09
RM26	0.86	1	4	0.23	1.37	0	0.21	1	1	0.67	0.12
RM22	0.94	0	2	0.10	0.89	0	0.13	1	1	0.83	0.05
RM14	0.61	1	3	0.37	0.85	0.12	0.17	0.31	0.86	0.8	0.06
RM171	0.91	0	2	0.15	1.09	0	0.13	1	1	0.79	0.07
RM335	0.53	4	7	0.42	1.21	0	0.11	0.98	1	0.82	0.06
RM44	0.44	2	8	0.64	1.86	0.17	0.38	0.55	0.78	0.52	0.23
RM49	0.86	3	5	0.23	0.9	0.11	0.15	0.28	0.84	0.78	0.07
RM50	0.39	2	5	0.60	1.3	0.03	0.2	0.84	0.95	0.71	0.1
RM55	0.8	1	4	0.31	1.42	0.11	0.23	0.53	0.63	0.21	0.95
RM60	0.95	0	3	0.09	1.35	0	0.14	1	1	0.7	0.11
RM80	0.79	1	9	0.36	2.19	0.3	0.45	0.32	0.61	0.42	0.34
RM105	0.37	0	5	0.63	1.67	0.19	0.35	0.44	0.71	0.48	0.27
RM118	0.66	4	7	0.52	1.27	0.07	0.18	0.63	0.89	0.7	0.11
RM126	0.73	2	4	0.38	1.1	0.02	0.06	0.68	0.96	0.88	0.03
RM129	0.58	3	6	0.41	1.42	0.07	0.23	0.7	0.9	0.67	0.12
RM133	0.48	5	7	0.43	1.18	0.09	0.09	0.07	0.67	0.64	0.14
RM144	0.81	1	5	0.30	1.37	0.03	0.25	0.86	0.95	0.66	0.13

RM152	0.5	3	8	0.60	1.71	0.09	0.32	0.71	0.87	0.55	0.2
RM134	0.82	0	4	0.26	1.41	0.15	0.24	0.38	0.64	0.42	0.35
RM136	0.81	2	7	0.29	1.77	0.5	0.31	-0.6	0.27	0.55	0.21
RM159	0.89	3	7	0.19	1.77	0.41	0.37	-0.1	0.47	0.52	0.23
RM161	0.92	0	4	0.14	1.44	0.08	0.26	0.68	0.88	0.64	0.14
RM162	0.9	0	3	0.17	1.21	0.03	0.14	0.8	0.95	0.73	0.09
RM184	0.84	4	13	0.28	1.5	0.33	0.27	-0.2	0.5	0.59	0.17
RM205	0.75	2	7	0.41	1.89	0.34	0.4	0.16	0.57	0.49	0.26
RM207	0.73	7	14	0.44	2.11	0.13	0.48	0.73	0.85	0.44	0.32
RM209	0.36	5	12	0.66	1.99	0.03	0.4	0.93	0.97	0.5	0.25
RM212	0.71	2	10	0.42	1.95	0.33	0.43	0.24	0.6	0.48	0.27
RM213	0.88	2	6	0.21	1.84	0.31	0.39	0.19	0.53	0.42	0.34
RM215	0.85	2	5	0.24	1.16	0.02	0.09	0.82	0.98	0.86	0.04
RM219	0.91	0	3	0.15	1.51	0	0.25	1	1	0.54	0.21
RM220	0.47	2	8	0.60	1.8	0.19	0.36	0.48	0.73	0.48	0.27
RM224	0.4	1	6	0.60	1.91	0.05	0.36	0.86	0.93	0.5	0.25
RM225	0.37	12	19	0.72	2.08	0.31	0.47	0.35	0.63	0.42	0.34
Mean	**0.66**	**2.18**	**6.49**	**0.41**	**1.54**	**0.12**	**0.27**	**0.62**	**0.83**	**0.59**	**0.2**

Where, PIC: Polymorphic Information Content; Ho: Observed Heterozygosity; He: Expected Heterozygosity; F_{is}: Wrights Fixation Index; F_{it}: Total Inbreeding Coefficient; F_{st}: Genetic differentiation.

of genomic DNA, 0.5 µl of each of the two primers (at a concentration of 10 mM), 1.75 µl of a 10X Taq buffer, 0.5 µl of a 2.5 mM dNTP mixture, 0.1 µl of Taq DNA polymerase and 3 µl of double distilled water. The temperature profile used for PCR amplification consisted of a 94 °C and 5 minutes preheating followed by 34 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C to 67 °C (depending on the annealing temperature of primer) for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min.

Polyacrylamide gel electrophoresis, SSR alleles scoring and analysis

The amplified PCR products (10 µl) were mixed with a 3 µl bromophenol blue loading dye and electrophoresed in an 8.0% polyacrylamide gel and detected using silver staining as described by [31]. The size of the most intensely amplified product was determined based on its migration in comparison with the size standard (50 bp DNA ladder). The different alleles of a marker were identified on the basis of their size or length in base pairs (bp) variation. Due to the co-dominant nature of SSR markers, the amplified bands representing the different alleles were scored as different genotypes. Thus, bands were recorded as (11, 22, 33. . .) to represent homozygous genotypes or (12, 13, 23. . .) to indicate the heterozygous genotypes. For each marker, '?' was used for missing data.

The number of alleles per locus, number of rare alleles, expected and observed heterozygosity and Fstatstic values like F_{is}, F_{it} and F_{st} were calculated using GenAlEx 6.502 [33]. Besides, the major allelic frequency and PIC of each marker were computed using Power Marker Version 3.25 [34]. To detect unique alleles, number of accessions having a specific allele was counted for each locus and alleles with a frequency of less than 5% were considered as unique alleles for a particular locus. According to [35], polymorphic information content (PIC) for each marker was also calculated.

Mathematically,

$Ne = 1/\sum pi^2$

Ho = Number of observed heterozygotes/total number of individuals

$$He = 1 - \sum_{i=1}^{n} pi^2$$

$$PIC = 1 - \sum_{i=1}^{n} pi$$

Fis = (Mean He - Mean Ho)/Mean He

Fit = (Ht - Mean Ho)/Ht

Fst = (Ht - Mean He)/Ht

Nm = [(1/Fst) - 1]/4

Key; Pi is the frequency of the i^{th} allele, $\sum pi^2$ is the sum of squared allele population frequency, Mean He is average He across the populations, mean Ho is average Ho across the populations and Ht is total expected heterozygosity.

Results

Overall allelic diversity

A total of 440 different and reproducible alleles were detected at the 67 microsatellite markers' loci. The number of alleles per locus generated by each marker ranged from 2 to 19, with an average value of 6.49. Among those SSR markers, the highest numbers of alleles (19, 14, 13, 13 and 12) were produced by RM225, RM207, RM184, RM206 and RM209, respectively and the smallest number of alleles (2) was produced for RM22 and RM171 (Table 5). The frequency of a major allele at each locus ranged from 34% (RM1) to 95% (RM60). And on average, 66% of the total alleles of the 67 SSR markers were common or major alleles.

For each SSR marker, effective number of allels (Ne), observed heterozygocity (Ho), genetic diversity (He), Wrights

fixation index (Fis), Total inbreeding coefficient (Fit), Genetic differentiation (Fst), and gene flow (Nm) were calculated and indicated in (Table 5). The effective number of alleles was 103.84 in total and per locus Ne varied from 0.85 (RM14) to 2.3 (RM1) with an average of 1.54. When all populations were pooled as one for each locus, the observed hetrozygosity (Ho) was in a range of 0 to 0.5 and 0.12 on average. From the 67 SSR markers-based study, level of genetic diversity (He) fluctuated between 0.06 (RM126) and 0.48 (RM207) with an average of 0.27.

In this study, the observed hetrozygosity (0.12) was actually less than the expected heterozygosity (0.27). From the entire microsatellite loci assayed in this study, the mean degree of genetic differentiation among populations (Fst), the Wright's fixation index (Fis), Total inbreeding coefficient (Fit), and gene flow (Nm) values were 0.59, 0.62, 0.83 and 0.2, correspondingly (Table 5). Here the average fixation index value (0.62) revealed significant deviation from Hardy-Weinberg expectations or significant heterozygote deficiency.

The degree of genetic differentiation (Fst) range of the whole loci was between 0.21 for RM55 and 0.88 of RM126. This very great mean Fst value (0.59), indicated the high levels of genetic differentiation among the sampled populations. Moreover, indirect estimates of gene flow from this very high Wright's Fst value showed the average number of migrants per generation across the *Oryza* species (Nm) as 0.2.

PIC values and unique alleles

Based on their allelic diversity and frequency, PIC values of the 67 SSR markers showed a very great variation. In this study, the highest PIC value (0.72) was recorded for RM225 and RM1. As shown on Table 5, 0.41 was the average PIC value and RM60 (0.09) and RM 22 (0.1) were rice SSR markers with the lowest PIC values. About 38.9% of the SSR markers used in this study showed PIC values higher than 0.5 and highly informative.

From the 440 alleles generated by the 67 microsatellite markers, 33.63% (148 of them) had a frequency ≤ 0.05 and were detected as rare alleles. The maximum number of unique alleles (12) were found in RM225 and the average of the rare alleles was 2.18 (Table 5). Moreover, 77.8% of the markers with higher PIC value (> 0.60) had at least one unique alleles. Generally, RM207 and RM225 were the most informative markers as, they identified rare alleles and produced the highest number of alleles (14 and 19), respectively.

Discussion

Microsatellites are PCR based DNA markers that are widely used in genetic diversity, varietal identification, and germplasm characterization of rice [36]. However, their distribution across the genome and level of polymorphism is quite different among each other [37]. Hence, their application on rice germplasm characterization and improvement will depend on reliability of the information they provide [6]. This comparative evaluation study of the rice SSR markers polymorphism also showed differences on their power of revealing genetic variation over diverse set of *Oryza* species and subspecies.

Differences in plant material composition, population size and species composition make direct comparisons of this study with others a bit irrational. This study however, estimates genetic diversity of each marker in terms of parameters like; the mean number of alleles, genetic diversity, PIC values and number of rare alleles over different *Oryza* species.

The mean number of alleles detected in the present study (6.49) is comparable to the number of alleles noted by earlier researchers on African rice [38]. According to [39], number of alleles in African wild rice (from 11 to 16 with an average of 14 alleles) is high. Thus, inclusion of large African wild rice materials in this study could lead to greater diversity than the results observed in previous studies showing 1-8 alleles with an average of 4.58 alleles for various classes of microsatellite [40] and also 3 to 9 alleles, with an average of 4.53 alleles per locus for 30 microsatellite markers [41]. Other than the diverse *Oryza* species assessed in this study, such variability on the number of detected alleles per locus might be associated to the markers' specificity [1].

In this study, all of the markers with highest number of allels (RM225 = 19, RM207 = 14, RM184 = 13, RM206 = 13 and RM209 = 12) have a dinucleotide repeat motifs of (CT)18, (CT)25, (GA)19, (CA)7 and (CT)18, respectively. Though 3 of those 5 markers were with (CT) motifs, [27] reported markers with repeat motif (GA) displayed high level of variation among the rice genotypes than markers with (CT) motifs. However, our results showed that markers having perfect dinucleotide repeat motifs irrespective of their dinucleotide type such as; (CT), (GA) or (CA)), are potentially best markers for molecular characterization and diversity analysis of different *Oryza* species.

The effective number of alleles in this study was 103.84 in total and per locus varied from 0.85 (RM14) to 2.3 (RM1) with an average of 1.54. Here the range was not wide as, the actual number of alleles varying between 2 and 19. This fact implicated the high influence of number of tested samples on the number of identified alleles and its insignificant impact on the effective number of alleles. It also reveals the higher reliability of effective number of alleles for practical genetic diversity analysis [42-44]. From the whole SSR markers assessed in this study, RM225, RM209, RM207 and RM84 produced Na greater than 10 and Ne greater than 2. Since such markers showed greater genetic diversity, they could be suitable tools for assessing genetic diversity within and among *Oryza* members. The highest levels of actual and effective number of alleles recorded in such locus also contribute to their great levels of expected heterozygosity (on average 0.46) [45].

However, all loci in this study except RM159 and RM184 showed a lower observed Heterozygosity (Ho) (mean = 0.12) than the expected Heterozygosity (He) (mean = 0.27), suggesting a clear shift from the Hardy-Weinberg equilibrium [46] and this shift can be attributed to forces akin to inbreeding within groups [47] or lack of distinctly isolated *Oryza* populations [46]. Such heterozygous deficiency or deviations from Hardy-Weinberg expectation were also indicated by the relatively high F_{is} value (0.62). The average F_{st}= 0.59, implicated 59% of the total genetic variation among populations. The mating

system and its consequence of high intrapopulation inbreeding (F_{is} = 0.62) could be major factors for the high total inbreeding (F_{it} = 0.83) [48]. In the present study, low value for the number of migrants per generation (*Nm* = 0.2) was estimated. In fact, pollen viability in the genus *Oryza* is in general limited to few minutes [49]. Thus, dispersal of whole plants by programmed abscission followed by floating downstream might be factors responsible for the observed *Nm* value.

According to [50], PIC value is a derivative of both allelic diversity and frequency. Sensitivity of genotyping method and location of primers in the genome largely affect PIC [6]. Thus, PIC value that reflects the allele frequency and diversity among accessions could be varied from one to another SSR locus [51]. In the present study, PIC values for the 67 SSR markers ranged from the lowest value of 0.09 (RM60) to the highest value of 0.72 (RM1 and RM225), with a mean of 0.41 (Table 5). This level of mean polymorphism (0.41) is consistent with the reported PIC value in previous works [2,52,53].

According to [54], markers with a PIC value more than 0.5 are regarded as highly polymorphic. About 38.9% of the SSR markers used in this study had PIC values exceeding 0.5 (Table 5). Such great PIC values maybe associated to the highly co-dominant expression or presence of multiple alleles [19]. The highly polymorphic markers implicated in this study are greatly informative for genetic studies and detection of more alleles at a specific locus [55].

These days, number of unique alleles in a population (private allelic richness) is largely considered for many conservation and population genetics applications [56], distinguishing different species and populations of a species [57] and inferring evolutionary history of a population [58]. As indicated in Table 5, the maximum and average numbers of unique alleles in this study were 12 (RM225) and 2.18 respectively. This wide variation in the numbers of private alleles might show the variable periods of genetic isolation during the evolutionary history of *Oryza* species [48]. Markers used in this study and their association with rare alleles could be utilized by plant breeders and geneticists for the marker assisted selection programs [5]. Moreover, possible relationship of such unique alleles with diverse quantitative trait locus (QTL) regions must be studied [51].

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