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Characterization of Genetic Diversity among Wild Rice Accessions using Genome specific In-Del Markers

Biswaranjan Rout[1 3], Sridhar M[1 2], Muralidhara, B[1], Kamal Nath Reddy[1], KR[1], Sundaram RM[1], Anantha MS[1], Senguttuvel P, Subba Rao, LV[1], Padmavathi G[1], Ranganath HK[1], Fiyaz AR[1], Jyothi B[1], Suvarna Rani, C[1], Kalyani MB[1], Bidyasagar Mandal[1] and Gireesh, C[1]*

[1]ICAR-Indian Institute of Rice Research, Rajendranagar, Hyderabad - 500 030, India

[2]Department of Genetics and Plant Breeding, College of Agriculture, PJTSAU, Rajendranagar, Hyderabad - 500 030, India

[3]Centre for Biotechnology, Siksha 'O' Anusandhan University, Bhubaneswar, Odisha - 751 003, India

*Corresponding author (email: giri09@gmail.com)

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Abstract

The genus *Oryza* contains 22 wild species and two cultivated species namely *O. sativa* and *O. glaberrima*. In the present study, 17 accessions belonging to 17 different wild species of rice characterized using 21 wild rice genome specific In-Del markers. A total of 115 fragments/alleles were detected among the 17 wild species. The overall number of alleles per locus ranged from three to 10 with an average of 5.47 indicating the existence of high level of diversity among the accessions. The size of the detected alleles produced using the In-Del primer sets ranged from 105 bp with respect to ch10-044G to 545 bp in ch03-363w. The differences in molecular size between the smallest and the largest allele for a given In-Del locus varied from 25 bp (ch02-342w) to 375 bp (ch05-109G). The PIC values for 21 In-Del loci varied from 0.4 (ch04-276G) to 0.85 (ch05-109G) with an average of 0.68. The overall H_e values ranged from 0.54 (ch02-342w) to 0.87 (ch05-109G) with an average of 0.72. The cluster analysis grouped the 17 accessions into three main clusters. Cluster I consisted of two subclusters IA with *O. glumaepatula, O. longistaminata*, *O. rufipogon* and subcluster IB consist of *O. meridionalis*, *O. barthii*, and *O. nivara*. Cluster II consist of *O. rhizomatis*, *O. officinalis* and *O. eichingeri* which originated in same officinalis complex in rice evolution. Cluster III was subdivided into two sub-clusters with IIIA consisting of *O. punctata*, *O.minuta*, *O. ridleyi* and *O. longiglumis* and IIIB consisting of *O. latifolia*, *O. australiensis*, *O. alta* and *O. grandiglumis*. The results of the study reveal that genetic diversity among the accessions from different wild species considered in the present study is moderate. The In-Del markers utilized in this study were able to distinguish 10 out of the 17 species of *Oryza*. Based on the results obtained it can be concluded that In-Del marker based analysis in consonance with morphological marker data would be more appropriate to ascertain the identity of wild rice.

Key words: Genetic diversity, In-Del markers, wild rice species.

Introduction

Rice is the staple food for more than one third of the global population (Chakravarthi and Naraveni, 2006). The genus *Oryza* contains 24 species, of which 22 are wild species and two are cultivated species namely *O. sativa* and *O. glaberrima* (Brar and Khush, 2003). The wild species are either diploid or tetraploid with 2n=24 or 48 respectively. The species of the genus *Oryza* are broadly classified into four complexes (Vaughan, 1994) *viz*., *Sativa*, *Officinalis*, *Ridley* and *Meyeriana* complexes (Table 1).

Table1. List of wild species of rice used in this study

Sl. No	Species	IRGC No.	Ploidy	Genome	Chromosome number
1	*Oryza nivara*	80435	Diploid	AA	24
2	*Oryza rufipogon*	81900	Diploid	AA	24
3	*Oryza barthii*	104290	Diploid	AA	24
4	*Oryza longistaminata*	103560	Diploid	AA	24
5	*Oryza meridionalis*	105283	Diploid	AA	24
6	*Oryza glumaepatula*	100969	Diploid	AA	24
7	*Oryza punctuate*	88824	Diploid & Tetraploid	BB, BBCC	24,48
8	*Oryza minuta*	93257	Tetraploid	BBCC	48
9	*Oryza officinalis*	80733	Diploid	CC	24
10	*Oryzarhizomatis*	103410	Diploid	CC	24
11	*Oryza eichingeri*	81804	Diploid	CC	24
12	*Oryza latifolia*	99580	Tetraploid	CCDD	48
13	*Oryza alta*	100952	Tetraploid	CCDD	48
14	*Oryza grandiglumis*	105157	Tetraploid	CCDD	48
15	*Oryza australiensis*	100882	Diploid	EE	24
16	*Oryza ridleyi*	100877	Tetraploid	HHJJ	48
17	*Oryza longiglumis*	100974	Tetraploid	HHJJ	48

A systematic study analysis of genetic diversity of wild rice accession is essential to exploit the inherent variability of wild rice for adding novel traits and to broaden the genetic base of rice cultivars. Several studies have advocated use of molecular markers for precise and reliable characterization of diversity of wild rice germplasm (Karkousis *et al*., 2003). Allelic diversity in the germplasm is assessed by polymorphic information content (PIC). The PIC is reflection of allelic diversity and frequency among the germplasm lines (Krupa *et al.,* 2017). The extent of genetic diversity in the population is assessed by expected heterozygosity (H_e). The H_e is the probability that, at a single locus, any two alleles, chosen at random from the population, are different to each other (Nei, 1987).

Molecular markers namely RAPDs, AFLPs, SSRs, have been employed in molecular characterisation of wild species of rice (Lu *et al.,* 2009). Insertions/deletions (In-Dels) markers have also been employed in several studies (Varshney *et al.,* 2007). In-Dels are easy to use, PCR-based, co-dominant, highly polymorphic and widely distributed throughout the genome (Pacurar 2012; Lv *et al*., 2013; Yamaki *et al*., 2013; Wu *et al*., 2014). Short sequence and homo nucleotide repeats in genome tend to accumulate In-Dels due to polymerase slippage during replication while frame shift In-Dels in coding regions can result in loss of function or non-sense mutation (Rockah-Shmuel *et al*., 2013). Yamaki *et al.,* (2013) have developed 22 In-Del markers specific to genomes of wild species of rice. These markers were suggested to be useful in characterization and identification of different wild species of rice. In the present study 17 wild species of rice were characterized with the 21 In-Del markers developed by Yamaki *et al*. (2013) to ascertain the species identity and for molecular diversity analysis.

Materials and methods:

Plant materials: A total of 17 wild rice accessions each one represents different rice species genome (Table 1), available at wild rice garden, ICAR-Indian Institute of Rice Research (ICAR-IIRR), Hyderabad.

Genomic DNA isolation and quantification: Total genomic DNA was isolated from 15 days old seedlings of wild rice plants following CTAB (Cetyl Try Methyl Ammonium Bromide) method as described by Zheng *et al.* (1991). The total genomic DNA was quantified using 0.8% agarose gel electrophoresis lamda (λ) Hind III DNA as standard. The DNA samples were normalized in $T_{10}E_1$ buffer (10 mMTris-HCl, 1 mM EDTA, pH 8.0) to get final concentration of 50 ng/ µl for PCR amplification.

In-Del markers*:* Oligonucleotide sequence of 21 In-Del markers (Yamaki *et al*., 2013) accessed at on the integrated rice science database, Oryzabase (http://www.shigen.nig.ac/oryzabase) and the primers were custom synthesised (IDT, USA) for further studies.

Polymerase chain reaction (PCR): The PCR reaction was performed as described in Yamaki *et al*. (2013) in a total reaction volume of 10 µl containing 2.5mM dNTP mix, 10 pmol each of forward and reverse Indel primers, 10x optimized Taq buffer and 1unit of *Taq* DNA polymerase. PCR was performed under conditions of 94^{0}C for 2min and a subsequent 35 cycles of 94 ^{0}C for 1min, 56 ^{0}C for 1min and 72 ^{0}C for 1min, followed by 72 ^{0}C for 2 min. The amplified PCR products were mixed with bromophenol blue and resolved by 3% Agarose gel prepared in 1X TAE buffer stained with ethidium bromide at 37^{0}C (10mg/ml) along with 100bp ladder. The gel electrophoresis was carried out for three hours at 100 volts. The gel was viewed under UV trans-illuminator in a gel documentation system (Medicare, GELSTAN, USA). Gels were analyzed for status of the genotype by In-Del markers using the software of the gel documentation system. Total bands present in each cultivar was detected from the gels and scored manually. Based on the molecular weights of the bands, 1, 2, 3… scoring was subjected for statistical analysis.

Data Analysis: Molecular profiles of wild species obtained from the present study were compared with findings of Yamaki *et al*. (2013) to ascertain species identity. The genotypic data generated from 17 different wild species, 21 In-Del markers were utilized for cluster analysis and a Dendrogram was generated using DARwin v 6.0 (Perrier and Jacquemoud-Collet, 2006) based on unweighted neighbour joining (UNJ) method followed by boost strap analysis with 1000 per mutations for total cultivars. To measure the informativeness of the markers, polymorphism information content (PIC) for each of the In-Del markers was computed according to the formula: PIC =1− Σ pi^2, Where pi is the frequency of the i^{th} allele of each locus (Botstein *et al.*, 1980). Genetic diversity parameters *viz.,* number of alleles (*Na*) and expected Heterozygosity (H_e) (Nei *et al.,* 1973) were evaluated using POP-GENE version 1.31 (http://www.ualberta.ca/,fyeh).

Results and Discussion

Wild relative of crop plants possess enormous genetic diversity for various biotic, abiotic and yield enhance traits. Utilization of wild relative of crop plants through conventional and molecular breeding approach is imperative to broadening the genetic base of crop cultivars. Molecular characterisation and genetic diversity analysis is important tools to understand how the species have evolved, their relatedness and distinct genomic signatures among the various accessions of wild species. The Genus *Oryza* consists of 22 wild relatives and two cultivated species constituting the genetically most diverse species of model crop plants. In the present study, 17 wild species of rice (Table 1) were characterised with 21 genome specific In-Del markers (Table 2) to assess the molecular diversity among the wild species of rice.

Characterisation of wild species

Molecular characterisation of wild species of rice using 21 In-Del markers revealed that, out of 17 species studies, the amplicon size of In-Del markers in ten species namely, *O. rufipogon* (IRGC 80435) varied from 122bp (ch02-308w) to 455bp (ch03-363w), Similarly for *O. barthii* (IRGC-104290) from 122 bp (ch10-044G) to 444 bp (ch-03-363), in *O. glumaepatula* (IRGC-100969) from 122bp (ch-10-44W) to 450bp (ch-03-363W), in *O. punctata* (IRGC-88824) from 117bp (ch-10-44G) to 433bp (ch-03-363W), in *O. officinalis* (IRGC-80733) from 110 bp (ch-10-44G) to 525 bp(ch-03-363w), in *O. rhizomatis* (IRGC-103410) from 125bp (ch-10-44G) to 545bp (ch-03-363W), in *O. alta* (IRGC-100952) from 128 bp (ch-10-44G) to 434bp (ch-03-363W), in *O. grandiglumis* (IRGC-105157) from 130bp (ch-10-044G) to 437 bp (ch-03-363W), in *O. australiensis*, (IRGC-100882) from 126bp (ch-10-44G) to 450bp (ch03-363w) and in *O. meridionalis* (IRGC-105283) from 115 bp (ch-02-044G) to 459 bp (ch-03-276G) with one allele per marker loci in all these 10 species studied. The molecular profiles of the ten species were similar to the findings of Yamaki *et al.* (2013) indicating the identity of species and utility of the In-Del markers in identification of ten wild species of rice.

Interestingly, the molecular profile of six species namely O. *longistaminata, O. longiglumis, O. minuta, O. eichingeri, O. latifolia,* differed from the report of Yamaki *et al.* (2013) with respect to either amplicon size or number of alleles per markers (Table 2). The findings from the present study need to be further confirmed with morphological data to ascertain the species identity. Importantly, this study also characterized, molecular profiling of *O. nivara* (IRGC-80435) accession with the In-Del markers, which was not characterized by Yamaki *et al*. (2013). Further, this information will be useful for identification of the *O. nivara* species.

Genetic diversity analysis

The amplicon size of the detected alleles of 21 In-Del markers among the 17 wild species of rice ranged from 105 bp with respect to the marker ch10-044G to 545 bp in the marker ch03-363w indicating the presence of large insertions and/deletions in the wild rice genome of different species. Similar kinds of results were reported earlier with a large difference in the number of repeats between the

different alleles for SSR primers by Adegbaju *et al.* (2015). A total of 115 alleles were amplified by the 21 In-Del markers on in different wild species of rice. The number of alleles per marker ranged from three to 10 with an average of 5.47 alleles (Table 2) indicating the presence of higher allelic diversity of In-Del markers among the accessions of wild species studied. This was comparable to the report of Rani *et al.* (2016) who reported average allele number of 4.3 per locus as high level of allelic diversity.

Table 2. The list of 21 In-Del primers used in the estimation of genetic diversity of 17 wild rice accessions, showing variation for number of alleles (AN), amplicon size (bp), PIC (polymorphism Information Content) and H_e (Genetic Diversity) values

S. No.	Primer Name	amplicon size (bp)	No. of Alleles	PIC	H_e Values Genetic diversity index
1	ch01-301w	347 – 421	6	0.74	0.78
2	ch02-308w	115 – 189	3	0.53	0.6
3	ch02-342w	200 – 225	3	0.49	0.54
4	ch02-343w	128 – 388	7	0.75	0.78
5	ch03-128w	271 – 324	4	0.66	0.71
6	ch03-173w	114 – 374	6	0.73	0.77
7	ch03-363w	427 – 545	6	0.77	0.8
8	ch04-276G	115 – 148	3	0.48	0.55
9	ch04-312w	148 – 203	4	0.68	0.73
10	ch05-067w	118 – 235	6	0.71	0.75
11	ch05-070w	234 – 378	6	0.77	0.8
12	ch05-109G	151 – 526	10	0.85	0.87
13	ch05-202w	127 – 280	4	0.59	0.65
14	ch05-277w	288 – 345	5	0.68	0.72
15	ch06-269w	259 – 394	8	0.79	0.81
16	ch06-300w	150 – 196	5	0.69	0.73
17	ch06-306w	106 – 200	7	0.77	0.79
18	ch07-233w	168 – 327	9	0.8	0.82
19	ch08-006w	228 – 295	5	0.76	0.79
20	ch09-037G	116 – 188	5	0.65	0.7
21	ch10-044G	105 - 135	3	0.55	0.62
Total			**115**	**14.44**	**15.31**
Mean			**5.47**	**0.68**	**0.72**

The marker ch05-109G showed maximum number of alleles (10 alleles) with the highest PIC value (0.85) and H_e index (0.87) followed by the marker ch07-233w with nine alleles, 0.80 PIC value and 0.82 H_e index. The Polymorphism information content (PIC) values provides an estimates of discriminating power of a marker based on the number of alleles at a locus and relative frequencies of these alleles (Chen *et al*., 2017). The PIC values of 21 In-Del markers in our study varied from 0.4 (ch04-276G) to 0.85 (ch05-109G) with an average of 0.68 while more than 40 per cent markers were in the range of 0.7 – 0.8 (Figure 1) indicating wide distribution of polymorphism among the markers. Hence these In-Del markers could be considered as highly informative and useful for genetic diversity studies. Nei's genetic diversity estimated by average expected heterozygosity (H_e). It is the probability that, at a single locus, any two alleles, chosen at random from the population, are different to each other Nei (1973). The overall H_e values ranged from 0.54 (ch02-342w) to 0.87 (ch05-109G) (Table 2) with an average of 0.72 while more than 50 per cent of the markers were in the frequency of 0.7-0.8 (Figure 1). The average H_e over all loci is an estimate of the extent of genetic variability in the population. H_e value will be maximum, when there is maximum number of alleles per locus. The average H_e in the study was 0.72 indicating the presence of high genetic diversity. The un-weighted neighbour joining (UNJ) dendrogram was constructed based on polymorphism data of the 21 In-Del markers using DARwin 6.0. The dendrogram classified 17 accessions into three main clusters (Figure 2). Cluster I consist of AA genome species of *Oryza* complex. The Cluster I was further classified into two sub-clusters IA with *O. glumaepatula, O. longistaminata*, *O. rufipogon* and sub-cluster IB consist of *O. meridionalis*, *O. barthii*, and *O. nivara*. Cluster II consist of *O. rhizomatis* (CC), *O. Officinalis* (CC) and *O. eichingeri* (CC) which belongs to *officinalis* complex with CC genome. Cluster III sub-divided into two with IIIA consist of *O. punctata (BBCC)*, *O. minuta (BBCC)* and also *O. ridleyi (HHJJ)*, *O. longiglumis (HHJJ)* while IIIB consist of *O. latifolia (CCDD)*, *O. alta (CCDD), O. grandiglumis (CCDD)* and also *O. australiensis* (EE). Maximum dissimilarity (Table 3) was observed between *O. rufipogon* and *O. grandiglumis* (0.732) possibly due to distant ancestry of the two wild species. Similar kind of results were observed by Kundur *et al.* (2015) who reported 37% of genetic variation for target region amplification polymorphism (TRAP) markers.

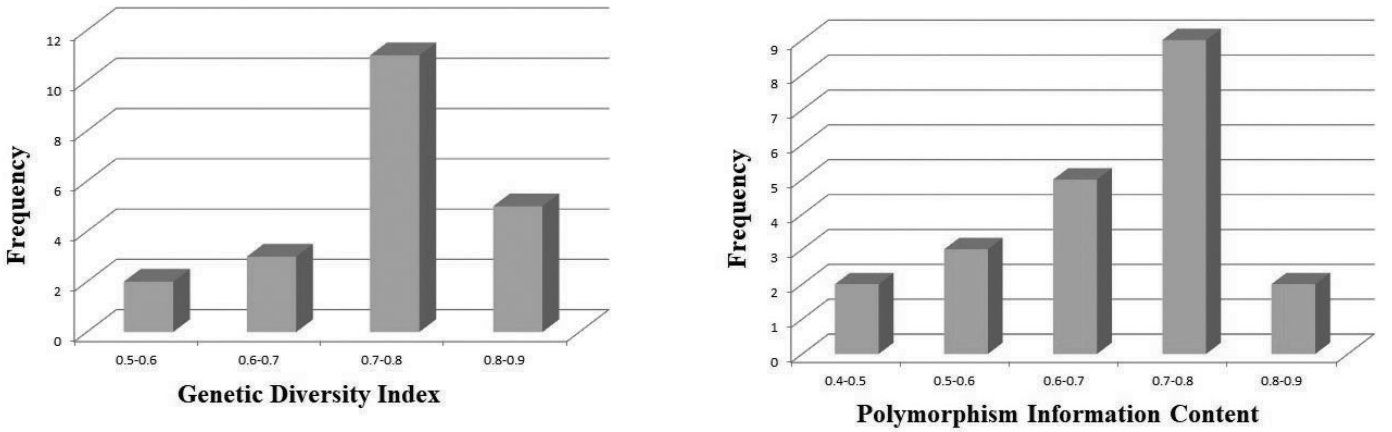

Figure 1. (a) Frequency distribution of Genetic Diversity Index and (b) Polymorphism Information Contents

Table 3. Jaccard Dissimilarity Matrix as obtained based on 21 In-Del markers

Genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2	0.95															
3	0.975	0.975														
4	0.95	1	1													
5	0.975	1	0.975	0.95												
6	0.975	0.785	0.978	0.933	0.978											
7	0.981	0.976	1	1	0.785	0.957										
8	1	0.978	1	0.795	0.885	0.847	0.978									
9	1	1	0.933	1	1	0.978	0.976	0.884								
10	0.753	0.966	0.975	0.884	0.785	1	1	1	0.975							
11	1	0.973	1	0.978	0.945	1	1	0.732	0.977	1						
12	1	0.845	1	0.951	1	1	1	1	1	0.976	0.978					
13	1	1	0.975	1	0.975	0.978	0.973	0.978	1	1	1	1				
14	0.975	0.975	0.952	1	1	0.845	0.981	0.978	0.975	1	1	0.878	0.95			
15	1	1	0.975	1	1	0.978	0.951	1	0.878	0.966	0.977	0.983	1	1		
16	1	0.878	0.967	1	0.945	0.883	0.963	0.978	0.993	0.976	1	1	1	1	1	
17	1	1	0.975	1	1	0993	1	1	0.971	0.884	1	0.963	1	0.975	0.975	1

1. *O. punctata*, **2.** *O. minuta* **3.***O. rhizomatis*, **4.** *O. ridleyi*, **5.** *O. longiglumis*, **6.** *O. nivara*, **7.** *O. barthii*, **8.** *O.grandiglumis*, **9.** *O. meridionalis*, **10.** *O. longistaminata*, **11.** *O. rufipogon*, **12.** *O. glumaepatula*, **13.** *O. latifolia*, **14.** *O. australiensis*, **15.** *O. eichingeri*, **16.** *O. alta*, **17.** *O. officinalis*

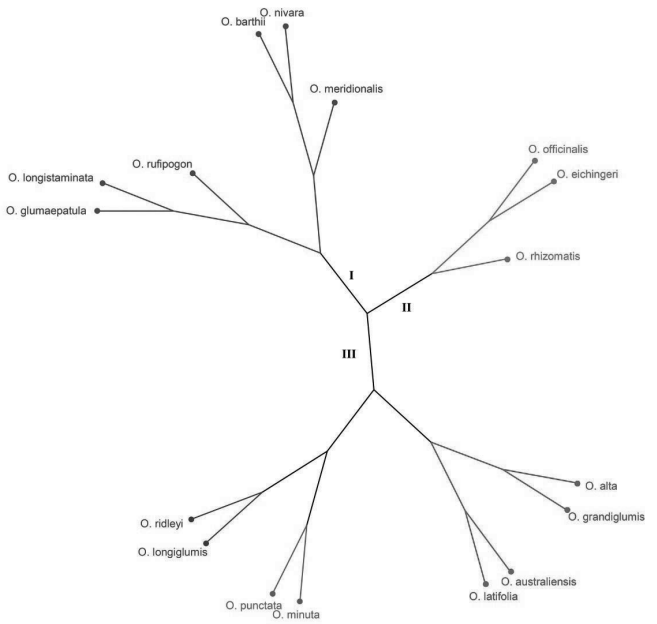

Figure 2. Un-weighted Neighbour Joining (UNJ) tree showing genetic relationship among 17 wild species for 21 In-Del markers

The In-Del markers used in the study were useful in distinguishing the most of wild species of rice. The molecular profile from In-Del markers along with morphological data should be used for discriminating and classifying the wild rice. In-Del markers were also useful in assessing the genetic diversity of wild rice. Nearly 11 species of wild rice genomes have been sequenced (www.Gramene.org). Using genome sequence information of wild species of rice, it is essential to identify unique genome signature to each genome of wild rice to further develop the robust and non-ambiguous DNA markers to distinguish the different species of wild rice without any perplexity.

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