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Molecular and morphological characterization of Indian rice hybrids

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Abstract

Commercial exploitation of hybrid vigour is a viable and practicable option to enhance productivity levels in rice. The success of hybrid rice in China has stimulated hybrid rice research in India and some promising rice hybrids were developed. The present study is assessments of the genetic diversity present in twenty eight F_1 hybrids released in India with almost all are based on WA cytoplasmic source. At phenotypic level, variation for important agro-morphological traits, grain quality was studied while at molecular level, the variation at nuclear level was examined using rice genomic SSRs and markers associated with fertility restoration (*Rf3, Rf4)* and ribosomal DNA sequences (ITS 1, ITS 2 regions) and the variation at the organelle genome was analysed with markers associated with the ORF100 region of the chloroplasts. The results suggest that the hybrids fall into a narrow range for both duration and plant stature and majority of the hybrids have long kernels. Though earlier reports suggest the presence of both *Rf3* and *Rf4* genes to restore fertility, the positive allele of *Rf3* was present in only 43 % of the hybrids while a different allele of *Rf4* restores fertility in Pusa RH10. The ITS1 region was more diverse compared to ITS2 region, none of the hybrids possess *japonica* type of organelle genome and the genetic base of the hybrids have not widened enough over years (31.7% since 1995). Though diversification of cytoplasmic male sterile source remains to be the high priority issue, the results suggest that widening of genetic base is crucial to enhance hybrid vigour and productivity in the next generation hybrids.

Keywords: Rice, Hybrid, Genetic diversity, ORF100, Fertility restoration.
Abbreviations: SSR_simple sequence repeat, PCR_polymerase chain reaction, WA_wild abortive, *Rf*_restorer fertility, ITS_internal transcribed spacer, IGS_intergenic spacer.

Introduction

Rice, the most important staple food crop, supports around 50% of the world population and is grown under a wide range of ecologies. The genetic diversity in the rice germplasm is quite large in comparison to other crop species. Of the two cultivated species, the widely distributed *O. sativa* is cultivated throughout the world while the other species, *O. glaberrima* is restricted to the African continent. *O. sativa* comprises three subspecies, *i.e., indica*, *japonica* and *javanica* that collectively represent the large reservoir of rice germplasm that include a variety of local landraces and cultivars (Khush, 1997; Lu et al., 2005; Garris et al., 2005). In addition, there are a number of wild relatives that provide potentially valuable resources for the improvement of cultivated rice (Khush, 1997; Ren et al., 2003). Despite the richness of genetic resources, reduced genetic base and the prevalence of only a small set of landraces in the breeding process had been the general approach for several crops species including rice (Souza and Sorrells, 1989; Dilday, 1990; Cuevas-Perez et al., 1992). During the green revolution era of 1970s, with the use of high yielding semi dwarf varieties, rice yields have witnessed a quantum jump. In the later years, with rice yielding stagnating, hybrid rice came into prominence as productivity levels can be increased significantly with its use. Commercial exploitation of hybrid vigour is one of the most significant applications of genetics in agriculture and it has not only contributed to food security but has also benefited the environment (Duvick, 1999). With the clear demonstration of its potential in China, hybrid rice technology is considered as a viable option to follow to meet the future rice production targets. As a result, hybrid rice varieties have been released in more than twenty countries and currently it has now become the primary means to increase the productivity levels of rice. In India, hybrid rice varieties covered an area of 1.5 million hectares (2010-2011) with more than 80% of the hybrid rice coverage was in North India (U.P, Bihar, Punjab, Haryana, Jharkhand and Chhattisgarh). The government of India has set a target of expanding the cultivation of hybrid rice to 25% of the area occupied by the crop by 2015 (Spielman et al., 2013). Of the two systems currently available for hybrid rice development, all the hybrids released in India so far, were developed employing the three line system that involves a male sterile line (A), a maintainer line (B) and a restorer line (R). Knowledge regarding the extent of variation present and genetic relationships among the existing hybrids are important considerations for designing the future hybrids. In the past, the characterization of diversity was carried out using morphological and biochemical markers which, in many cases, lack the resolution power to reveal the distinct polymorphisms and to differentiate between closely related genotypes (Alan, 2007). The present study is an attempt to assess the diversity present in twenty eight released hybrids. The variation present in both important agro morphological traits and grain quality was studied and for the molecular analyses, genome wide SSR markers, markers associated with fertility restoration (*Rf3*, *Rf4*), ribosomal DNA regions

Table 1. Analysis of variance for different agro-morphological traits in the hybrids.

Trait	Mean	Min	Max	MS	LSD5%	CV5%
Duration (days)	130.1	96.0	143.0	638.703	1.39	0.9
Plant height (cm)	106.1	83.6	134.8	688.362	3.92	3.0
Culm number	10.9	8	17	22.258	3.22	23.6
Panicle length (cm)	27.4	22.4	31.4	32.008	1.97	5.7
Leaf length (cm)	40.7	30.0	49.4	121.213	5.28	10.4
Leaf width (cm)	1.1	0.9	1.3	0.499	0.15	11.0
Ligule length (cm)	2.1	1.7	2.7	0.428	0.41	15.4
Flag leaf length (cm)	33.2	23.2	42.8	111.940	5.26	12.6
Flag leaf width (cm)	1.4	1.1	1.8	0.773	0.12	6.9
Grain length (mm)	9.6	8.1	11.7	4.117	0.76	6.3
Grain width (mm)	2.8	2.4	3.3	0.252	0.34	9.9
L/B ratio	3.5	2.8	5.0	0.990	0.58	13.1
Yield (t/ha)	4.1	2.5	6.7	0.509	0.52	10.1

MS: Mean square; LSD least significant difference of mean; CV coefficient of variation (F-pr) Probability value < 0.001

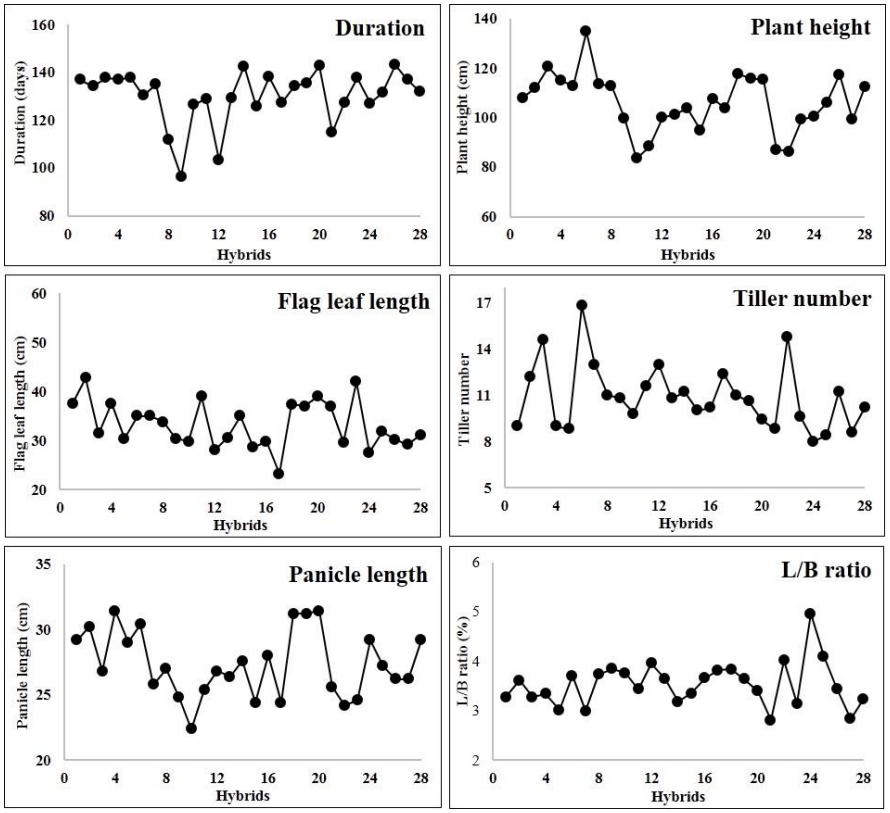

Fig 1. Variation for different morphological traits in the hybrids.

(*r*-DNA), and ORF100 region of the chloroplast genome were employed

Results

Variation for different morphological traits

Significant variation for agro-morphological characters like leaf length (30.0-49.4 cm), leaf width (0.9-1.3 cm), ligule length (1.7-2.7 cm), flag leaf length (23.2-42.8 cm), flag leaf width (1.1-1.8 cm), tiller number (8-17), panicle length (22.4-31.4cm), grain length (8.1-11.7 mm) and grain width (2.4-3.3 mm) was observed in the hybrids. The plant height and the duration were in the range of 83.6-134.8 cm and 96-143 days respectively (Fig.1). Among the hybrids, 82.1% were intermediate for blade pubescence character and around 7% of the hybrids have purple pigmentation at the base. Three different types of leaf angle were observed i.e. intermediate (67.9%), droopy (17.9%) and erect (14.2%). Intermediate type of culm angle was observed in 75% hybrids while the erect type was observed in 25% of the hybrids. In case of leaf angle, both intermediate (53.3%) and erect (46.7) types were present in equal proportions. Panicle exertion varied among the hybrids from poor (67.8%) to full exertion (10.7%) while the spikelet sterility varied from 11% (PA 6129) to 52 % (VNR 204) (Table 1). The analysis of variance suggests the presence of significant differences in the hybrids for all the traits.

Molecular marker analysis

Of the two genes associated with fertility restoration, the positive allele of *Rf4* was present in all the hybrids except one (PusaRH 10) where a different allele was observed. The positive allele of *Rf3* was present in only 43 % of the population (Fig.2). The hybrids showed high level of variation for ITS 1 sequence and eight different types of allelic combinations were detected. Of the different types

observed, type 5 (subunit) was the most predominant (40%) while 33.3% of hybrids belongs to type 4. The hybrids US 312, JKRH 3333, RH 1531, PA 6444, PA 6201 and CRHR 32 possess alleles of type 1, 2, 3, 6, 7 and 8 respectively. Compared to the ITS 1 region, the level of variation was low for ITS 2 region and only 5 different types were recorded with 60.0% of the hybrids belong to group 2 type and 20 % to group 1 type. The hybrids PA 6444 and PA6201 belongs to type 3 while PAC837 and PA 6129 belongs to Type 4 category. For the ribosomal intergenic spacer region (IGS) sequence, two different types of subunit combinations were recorded. The hybrids, JKRH3333 and PAC837 were classified as type 1 group (600 bp) while 86.7% of the hybrids had two amplicons (450 and 600 bp). In the ORF100 region of the chloroplast genome, a 69 base pair deletion, a feature specific to *indica* rice was detected in all the hybrids.In case of aroma, when tested with markers specific to *badh2* gene, 10 hybrids were homozygous for non-aromatic allele, one hybrid (Pusa RH10) was homozygous for the allele that confers aroma while in the rest of the hybrids, and the presence of both alleles was observed (Fig.2). Of the 60 SSR markers employed, 43 markers were found to be polymorphic among the hybrids. These markers amplified a total of 125 alleles and the average number of alleles per locus was 2.91 in the range of 2-5. The size of amplified PCR products were in the range of 50-480bp and the size difference between the smallest and the largest allele for a given locus varied from 5-110 bp. The *PIC* value ranged from 0.191 (RM463) to 0.871 (RM8213) with an average of 0.639. The number of unique alleles per locus varied from 1 to 3. The hybrid Ajay can be identified with a unique allele (360 bp) when amplified with the marker RM171 while INDAM200-017 showed unique alleles with markers RM311 (320bp), RM6321 (180bp) and RM1106 (220bp) and can be easily distinguished from other hybrids.

Genetic relationships among hybrids

The dendrogram constructed based on the genetic distance/similarity of thirteen agro morphological traits, grouped the hybrids into two major clusters A and B at 0.55 level (Fig.3). Twenty four hybrids were grouped into cluster A while cluster B had only four. Both the clusters (A and B) were further subdivided into two sub clusters each. Twenty three hybrids were grouped into sub cluster AI and cluster AII had only Pusa RH 10. The sub cluster B I contain three hybrids and sub cluster B II contain only JKRH 2067. The dendrogram constructed based on SSR data, grouped the hybrids into two major clusters A and B at 0.55 level (Fig.4) with twenty seven hybrids in cluster A while cluster B contained only INDAM200-017. The cluster A was further subdivided into five sub clusters at 0.67 level. The CRRI bred hybrids Ajay, Rajalaxmi and CRHR 32 were grouped in the same sub cluster (A-V) and sub cluster A-III had 14 hybrids in it. The similarity index between the hybrids ranged between 0.393 (JKRH3333, INDAM200-017) to 0.932 (PHB71, DRH775) with an average of 0.665. When the hybrids were compared for the levels of diversity in different five year period, the hybrids of different periods were having close similarity (Table 2). Genetic distance between each pair of five year interval periods suggest that it was higher between the released hybrids of 1995-2000 and 2001-2005 (0.2016) period while the value was lower among the hybrids developed in the periods of 2006-2010 and 2011 onwards (0.0366) in the year of release wise group (Table 2). The genetic diversity parameters within five years interval period estimated through separate analysis of year of release wise *indica*ted that *Na* value were increased by 35.7% from 1995 to till date. Further the genetic diversity value *(He)* was also found to be in the increasing trend by 31.7% (Table 3). Bayesian analysis of population structure using the model-based approach of Pritchard et al. (2000) with the admixture model provided support for the existence of genetic structure among the rice hybrids in India (Fig,5). The analysis of population structure distinguished the population with a highest *K* value (K=2) of 65.85. The population structure grouped the genotypes in to two subpopulations i.e. POP 1 with a membership percentage of 34.8% while POP 2 with 65.2%. The genotype INDAM200-017 was found to be a mixed individual with a probability membership of < 60%. The fixation index (F_{ST}) values of subpopulations were 0.3323 and 0.0030 for POP 1 and 2 respectively, while the pairwise allele frequency divergence value between the subpopulations was 0.0655 (Table 4).

Discussion

The objective of this study was to estimate the extent of genetic diversity present at both morphological and molecular levels and also to understand their genetic relationships in a set of twenty eight hybrid rice varieties released in India. Of the hybrids tested, most of hybrids had semi dwarf plant stature, a preferred plant type for recording higher yields since the green revolution era with the lone exception being TNAURHCO-4, a hybrid with a tall plant type. The data suggests that features like shy tillering plant type, heavy panicles with high spikelet number and erect flag leaf, proposed for new plant type (NPT), were not incorporated in these hybrids to realize higher yields though several hybrids had semi dwarf plant type. Panicle exertion remained poor in most of the hybrids which warrants incorporation of *eui* gene (s) associated with the upper internode elongation to minimize the yield losses that occur due to poor exertion of the panicle (Librojo and Khush, 1986; Yang et al., 1999). As most of the hybrids belong to medium (130-140 d) to medium early medium (120-130 d) duration, efforts are needed to address the late (>140 d) and early duration (~ 100 d) groups of rices as hybrids of these durations will have a greater impact in enhancing rice production in the country. However, hybrid seed production programs for these ecologies need significant improvements as seed costs may become prohibitive. The shift to semi dwarf stature along with high response to fertilizers, a major feature of modern rice varieties, is also extended to hybrid rice varieties. One of the major problems that need immediate attention in hybrid rice development is the spikelet sterility which was observed in the range of 11-52 %. The higher levels of sterility observed in the hybrids grown under ideal conditions in a normal wet season suggest the unstable nature of several hybrids. Use of highly stable CMS lines, new CMS sources along with identification of genes that can ensure full restoration of fertility and extensive use of *eui* gene(s) can address the problem. In majority of the hybrids, the kernels were of long slender type with the L/B ratio of around 3 *indica*ting the efforts made in the area of grain quality in the hybrids. However, the presence of the allele for aroma in several hybrids in heterozygous state suggests the production of a mixture of aromatic and non-aromatic grains in the produce of some hybrids, a factor that critically influences the market value of the produce. The presence of aroma allele suggests the use of IR 58025 A, a CMS line with WA cytoplasm and the results suggest that few hybrids were developed using a non-aromatic CMS lines. It is surprising to see that in only 22% of the hybrids, fertility restoration

Table 2. Nei's unbiased measures of genetic distance among the hybrid rice varieties developed at different intervals (five year period).

Year of release	1995-2000	2001-2005	2006-2010	2011 onwards
1995-2000	****	0.8175	0.8721	0.8695
2001-2005	0.2016	****	0.9381	0.9264
2006-2010	0.1368	0.0639	****	0.9641
2011 onwards	0.1398	0.0764	0.0366	****

Genetic identity (above diagonal) and genetic distance (below diagonal)

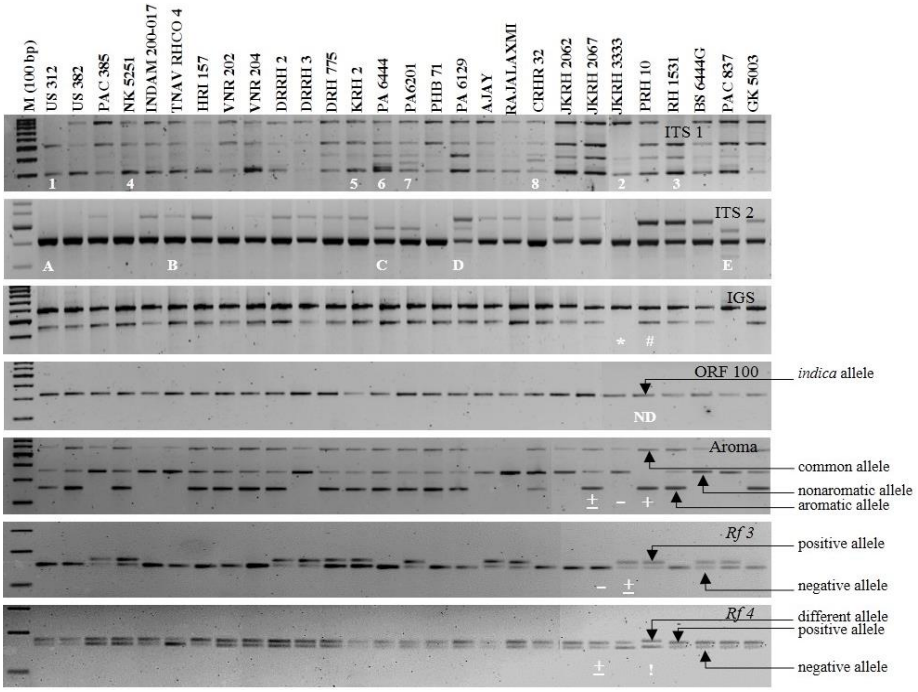

Fig 2. Gels illustrating allelic variation among hybrids with nuclear markers (ITS I, ITS II, IGS), aroma, fertility restoration (*Rf3*, *Rf4*) and organelle marker (ORF 100)ITS1- 1:*300+505bp*, 2-*300+400+900bp*, 3:*300+400+405bp*, 4:*300+505+900bp*, 5:*300+400+505+900bp*, 6: *300+320+340+400+500+900bp*, 7: *300+340+400+505+900bp*, 8: *300+380+400+505+900bp*; ITS2 - A:*405bp*, B:*405+550bp*, C:*405+495bp*, D:*405+495+550bp*, E:*350+405+495bp*; IGS - *:*600bp*, #:*450+600bp*; ORF100- ND: *indica*; Aroma- +:*aromatic*, -:*nonaromatic*, ±:*heterozygote; Rf3* & *Rf4* - ±:*heterozygote*, -: *absent*, !: *other allele.*

was controlled by both Rf3 and Rf4 while Rf4 could restore in 57% of the hybrids individually without the involvement of Rf3 suggesting the independent restoration ability of Rf4. Though both Rf4 and Rf3 were known to be associated with fertility restoration in rice and Rf4 was reported to be superior to Rf3 in fertility restoration (Yao et al., 1997) and this study records the independent restoration ability of Rf4 (in the absence of Rf3) in several Indian hybrids. The total dependence on these two fertility restoration genes and WA cytoplasm may lead to genetic vulnerability of the hybrids in the long run and diversification of cytoplasm and identification of new RF genes should be accorded highest priority in the hybrid breeding programs. The SSR analysis revealed only 2.91 alleles (range 2 to 5) per locus which is quite low compared with earlier reports for the worldwide collection (range 2 -11, mean = 6.3) and other large scale studies (range = 3-17, mean = 7.4) (Olufowote et al., 1997; Yu et al., 2003) but quite comparable to values reported for studies performed on smaller germplasm sets (Cho et al., 2000; Hashimoto et al., 2004; Siwach et al., 2004). The present study also revealed a low level of genetic diversity between the hybrids as evidenced by Nei's analysis. The model-based clustering method implemented in the STRUCTURE software also suggested the existence of two major groups in the released rice hybrids of India. Our results support the reports of narrow genetic variation in Japanese (Hashimoto et al., 2004) and Korean rice cultivars (Song et al., 2002 for which a narrow genetic base has been documented by means of SSR markers. One of the main reasons can be the use of IR 58025 A (WA type male sterility) in most of the hybrids. Excessive breeding emphasis in this direction given during the last fifty years knowingly or unknowingly has led to some sort of genetic uniformity among the currently cultivated high yielding varieties (Choudhary et al., 2013). The same was evident from the analysis of the genetic diversity between the hybrids developed during different five year periods as there is close similarity between them. Higher levels of diversity was observed in the early generation hybrids (first decade) than in the hybrids generated in the later years. It is interesting to find an O.glaberrima type IGS region in one of the hybrids suggesting the diverse materials used (Chang et al., 2010). Though it was proposed to use the indica-japonica derivatives to generate higher levels of heterosis, none of the hybrids developed so far did not have japonica genome, as indicated by the presence of deletion type of ORF 100 region in the chloroplast. Our results also suggest that it is essential to broaden the genetic base of the hybrid rice varieties of India to enhance the productivity levels. There is still a tremendous amount of unexploited genetic diversity in the primary gene pool of rice that can be employed for enhancing the diversity in the hybrids and their productivity under diverse agro ecological conditions (Guimaraes, 2000; Ali et al., 2006; Lafitte et al., 2006). Wild species of Oryza, the

Table 3. Summary statistics of genetic diversity parameters of major Indian hybrid rice cultivars.

Year of release	*Na*	*Ne*	*Ho*	*He*	*I*
1995-2000	1.6744	1.6000	0.4884	0.3140	0.4431
2001-2005	2.6047	2.0137	0.4302	0.4483	0.7377
2006-2010	2.8140	2.1439	0.4228	0.5066	0.8280
2011 onwards	2.6047	2.0207	0.4186	0.4597	0.7496

Na: Number of alleles; *Ne:* Number of effective alleles; *Ho*: Observed heterozygosity; *He*: Nei's genetic diversity; *I*: Shannon Index

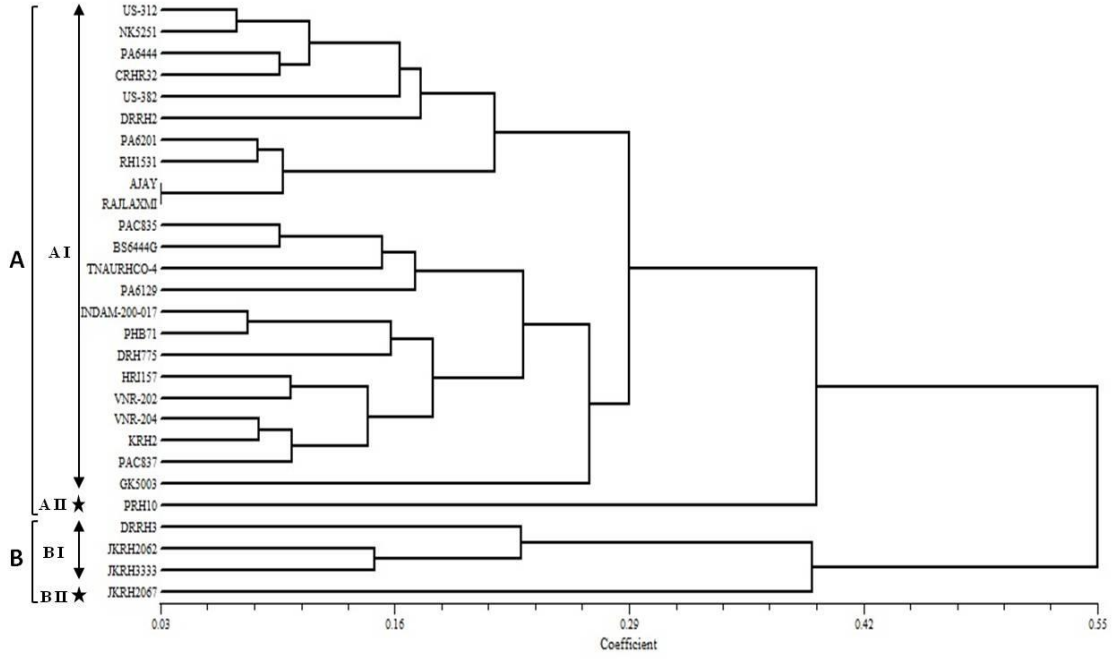

Fig 3. Dendrogram generated based on the dice similarity coefficient of agro morphological characters.

Table 4. Population statistics of the estimated clusters.

Population*	Membership (%)	F_{ST}	Average distances	Allele frequency divergence
POP 1	34.8	0.3323	0.4101	0.0655
POP 2	65.2	0.0030	0.5129	

**POP 1* and *POP 2* are estimated subpopulations

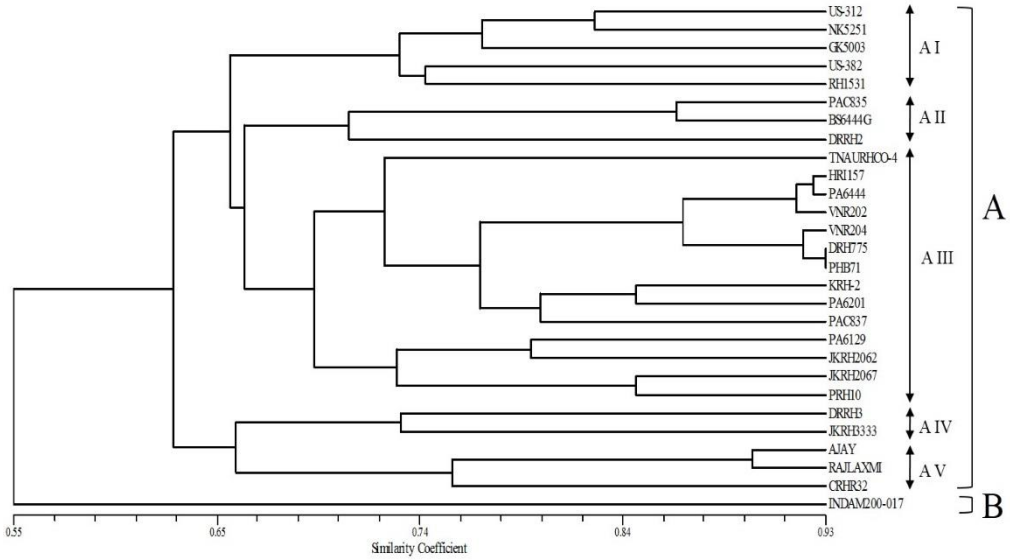

Fig 4. Dendrogram obtained by UPGMA analysis based on SSR generated data showing genetic relationship among the hybrids.

Table 5. The list of primers used in the study associated with different traits.

Area of study	Primers	References
Restorer fertility	Rf3 (RM10305)	Shah et al., 2012
Restorer fertility	Rf4 (RM6100)	Singh et al., 2005
Internal transcribed spacer	ITS 1 (P1, P2) & ITS 2 (P3, P4)	White et al., 1990
Ribosomal intergenic spacer	IGS	Fujisawa et al., 2006
Chloroplast DNA	ORF100	Prathepha, 2008
Aroma	ESP, EAP, IFAP, INSP	Bradbury et al., 2005

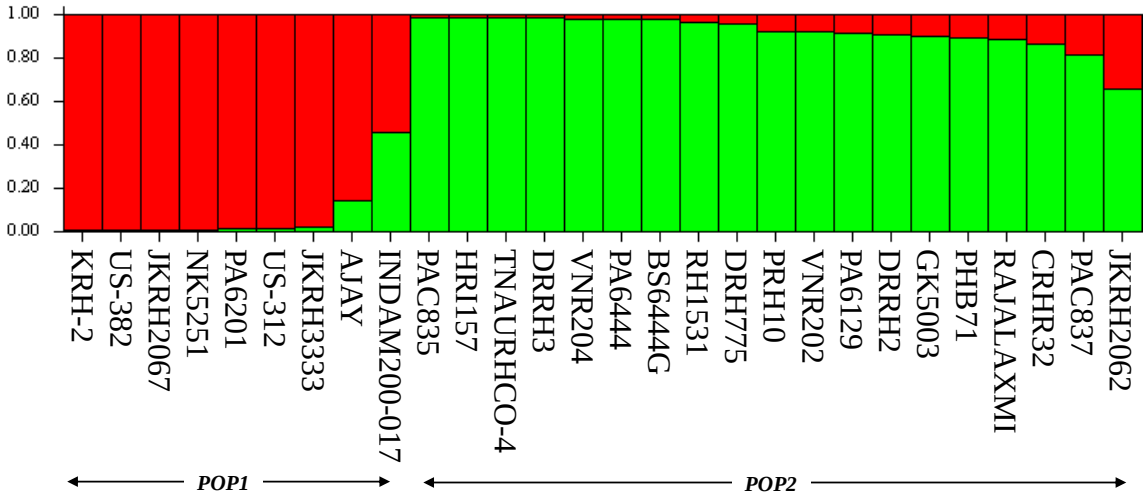

Fig 5. Population structure of hybrid rice varieties. The optimal value of *K*=2

secondary gene pool, a potential source of new alleles for improving yield, quality, and resistance/tolerance to various stresses, are also of immense value (Xiao et al., 1998; Moncada et al., 2001; Ahn et al., 2002; Thomson et al., 2003; McCouch, 2004; Kovach and McCouch, 2008).

Materials and methods

Plant materials

Twenty eight released hybrids, developed from both public and private sector were selected for the study (Table S1). Since the hybrid seed sells at a premium rate with high economic returns, it is assumed that a wider range of variability was used in their development to make the hybrids novel and highly productive.

Field evaluation

The hybrids were evaluated under field conditions at the experimental farm of Central Rice Research Institute, Cuttack. Thirty-days-old, nursery grown, seedling of the hybrids were transplanted in a complete randomized block design with two replications with 15 X 20 cm (within and between lines) spacing and the data was collected on the agro morphological traits and yield. The morphological data was collected as per DUS guidelines prescribed for rice.

DNA Markers

Of the nuclear sequences, three specific regions of the ribosomal DNA i.e. ITS1, ITS2 and IGS, sequences related to *Rf4* and *Rf3*, the genes associated with fertility restoration, a specific sequence of the ORF 100 region of chloroplast DNA, were amplified to study the relationship between the hybrids (Table 5) (Shah et al., 2012; Singh et al., 2005; White et al., 1990; Fujisawa et al., 2006; Prathepha, 2008; Bradbury et al., 2005) Further, sixty rice microsatellite markers, five representing each chromosome of rice were used in the study to study the variation at genomic DNA level.

DNA isolation and PCR assays

The genomic DNA was extracted from 10 days old rice seedlings as per Dellaporta et al. (1993). PCR analysis was performed with 0.2 µl Taq DNA polymerase (5U/µl) (Biotools), 1 µl of genomic DNA 10 ng/µl, 1 µl of 10X buffer (Biotools), 0.5 µl of dNTPs (2.5 mM) and 1µl of each primer pair in a total volume of 10 µl. PCR was performed using thermal cycler (Applied Biosystems) following the PCR protocols reported earlier with necessary modifications. The amplified products were separated in 3% metaphor gel and visualized under phospho imager system after staining with ethidium bromide. The sizes of the amplified fragments were estimated visually using 100bp DNA ladder as size standard. Only clear and unambiguous bands of markers were scored. Markers were scored for the presence (1) or absence (0) of the corresponding band among the genotypes.

Statistical analysis

The polymorphism information content (PIC) for each SSR marker was calculated using the formula: $\mathrm{PIC}i = 1 - \sum_{j=1}^{n} (Pij)^2$ where n is the number marker for marker i and Pij is the frequency of the jth allele of marker i (Anderson et al., 1993). Genetic diversity parameters viz., number of alleles *(Na)*, observed heterozygosity *(Ho)*, Shannon Index *(I)*, Nei's genetic diversity index *(He)* (Nei, 1973) and unbiased pairwise genetic distance were evaluated using POPGENE v 1.32 (http://www.ualberta.ca /fyeh). The clustering analysis was done using NTSYS-pc (Version2.02) (Rolf, 1998) to construct unweighted pair group using arithmetic averages (UPGMA) dendrogram based on dice

similarity coefficient of the genotypes. ANOVA implemented in the software of INDOSTAT was used to conduct the analysis of variance. The structure analysis (on the rice microsatellite marker data) was done with the software STRUCTURE (Pritchard et al., 2000). With 50,000 burning periods, 50,000 replicates and five runs, the admix model was used to estimate each *K* value, with ten independent runs from *K* = 1 to 10. Delta *K* was estimated as described by Evanno et al. (2005).

Conclusions

With the hybrid rice gaining ground in several Asian countries, the genetic diversity present at both morphological and molecular levels in the hybrids developed so far in India was carried out. The hybrids are in a narrow range in both duration and plant type and none of the hybrids have a *japonica* type of organelle genome. The results suggest diversification of cytoplasmic male sterile source is an immediate necessity to avert crop vulnerability. In addition, incorporation of favourable *japonica* alleles is likely to enhance the hybrid vigor and to minimize sterility, extensive use of *eui* gene(s) is suggested.

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