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Morpho-molecular diversity analysis of indigenous rice (*Oryza sativa*) germplasm

SARITA KUMARI[1], SATYAN[1], V K SHARMA[1], ASHUTOSH SINGH[2] and SUMEET KUMAR SINGH[3]*

Dr. Rajendra Prasad Central Agricultural University, Samastipur, Bihar 848 125, India

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ABSTRACT

Wild relatives of crops serve as the reservoir of kingpin genes related to various agronomic traits that play a significant role in crop improvement for sustainable agriculture. However, bequeathing these genes from wild rice has often been compromised due to climate change and anthropogenic activities posing serious threats to their natural habitats, leading to erosion of diversity. The experiment was carried out during 2021–2023 at Dr. Rajendra Prasad Central Agricultural University, Samastipur, Bihar aimed to study the utility of ISSR markers for genetic diversity analysis, population stratification, and identification of suitable donors for using in breeding programme for yield and climate resilience in rice (*Oryza sativa* L.). The results suggested that ISSR markers are highly informative for diversity analysis in rice. These markers showed a high level of polymorphism (85.40%) with high Polymorphic Information Content, Marker Index, and Resolving Power. ISSR markers; UBC807, UBC812, and UBC841 were identified as highly informative markers for rice. Two subpopulations were identified based on parametric and non-parametric approaches for population characterization, having the potential to be used in marker-trait association studies. Germplasms NKSWR 372, NKSWR 457, NKSWR 126, and NKSWR 245 exhibited superior agronomic performance comparatively. These elite genotypes may be utilized as potential donor for various rice improvement programmes.

Keywords: Genetic diversity, ISSR, Population structure, Principal component biplot analysis, Wild rice

The geographical diversity of India has contributed to intricate crop evolution and adaptation in response to progressively changing climatic conditions. Wild rice (*Oryza* spp.) growing across all the fifteen agro-climatic zones of the country serves as gold mines for genes regulating agronomically important traits and wider adaptability against biotic and abiotic stresses (Kumari *et al.* 2017, Kumari and Singh 2018). However, there is very limited literature available on the genetic background of these local wild rice. The collection, multiplication, and conservation of wild rice accessions from different agro-climatic zones is inevitable work for the nation to combat the future inescapable demand of the growing population under climate change regimes. *Oryza rufipogon* and *Oryza nivara* are the two progenitors of Indian cultivated rice (Samal *et al.* 2018). However, recent studies consider them as *O. rufipogon* Griff. Species complex (ORSC) (Kim *et al.* 2016). More than 300 accessions of *Oryza rufipogon* and 700 accessions of *Oryza nivara* are being conserved at the National Gene Bank. The NIPB, New Delhi, has also initiated efforts to collect various accessions of ancient rice from different agro-climatic zones (Tripathy *et al.* 2018). The characterization of this collected germplasm identified three subgroups based on pSINEs, SSR, and SNP markers (Singh *et al.* 2018, Kumari *et al.* 2021a). However, these marker systems are codominant, highly polymorphic, and locus-specific, but they need extensive input and cost for the development of locus-specific information in a particular species. Thus, a low-cost marker system needs to be developed that is highly polymorphic for a species and provides multi-locus comparison irrespective of their genetic background. Similar systems have been developed for the SSR named hypervariable SSR (HvSSR) markers for rice that can compare more than 700 rice accessions with only 33 SSR markers (Singh *et al.* 2016). The advantage of ISSR markers over SSR is that a single ISSR primer binds to a number of loci, and genetic polymorphism in the genome of unknown species may be observed at a much lower cost. The ISSR method was proven especially useful in the Poaceae family for the analysis of nearly isogenic lines (Akagi *et al.* 1996) and in the differentiation of rice varieties (Parsons *et al.* 1997). Multi-locus binding also ensures an unbiased

[1]College of Basic Sciences and Humanities, Rajendra Prasad Central Agricultural University, Samastipur, Bihar; [2]Centre for Advanced Studies on Climate Change, Rajendra Prasad Central Agricultural University, Samastipur, Bihar; [3]Post Graduate College of Agriculture, Rajendra Prasad Central Agricultural University, Samastipur, Bihar. *Corresponding author email: sumeet.singh@rpcau.ac.in

comparison of the genome. Thus, the identification of a set of ISSR primers with high polymorphism percentage and information content will reduce the cost and provide an ideal system for genetic diversity analysis. The present study was conducted with the intention to examine the role of ISSR primers for diversity analysis in rice and identify the informative ISSR primers, in addition to the identification of genotypes with higher agronomic performance based on principal component biplot analysis.

MATERIALS AND METHODS

Materials: The experiment was carried out during 2021–2023 at Dr. Rajendra Prasad Central Agricultural University, Samastipur, Bihar. A total of 22 diverse wild rice accessions were collected from NIPB, New Delhi (Supplementary Table 1), and 15 ISSR primers were used for the present study (Table 1).

Estimation of morphological variations: The accessions were grown in the field during rainy (*kharif*) season 2022, in a randomized block design (RBD) with two replications. Standard agronomic practices were performed to raise the crop. Agronomically important traits; plant height (PH), tiller number (TN), days to 50% flowering (DTF), panicle number (PN), panicle length (PL), seed yield/plant (SY), and biological yield/plant (BY) were recorded from ten plants/plot in each replication and mean data were used for the analysis (Supplementary Table 2). The variation among the genotypes for the mentioned agro-morphological trait was estimated through analysis of variance (ANOVA). Principal component analysis was done using phenotypic data, and their contribution to the phenotypic variance was estimated using various packages of R programme.

Estimation of genetic diversity

Genomic DNA isolations: The young leaves (0.1 g) of ten different plants of each wild rice accession were collected, and DNA was isolated using a modified CTAB-DNA isolation method (Doyle and Doyle 1987). The quality and quantity of isolated DNA were assessed through 0.8% agarose gel electrophoresis and a nanodrop spectrophotometer. An equal amount of DNA from each leaf was bulked.

ISSR genotyping: The isolated DNA from the sample was diluted and used for PCR analysis using master mix 1X concentration of buffer with 0.5 μM concentration of ISSR primers, and the PCR reactions were performed. The reproducible and consistent bands were only used for analysis. Genotyping data were scored as presence (1) and absence (0) of the band for each primer binding site that is regarded as a locus. The annealing temperatures were standardized to meet the number of bands up to 10 loci for each ISSR primer. The Resolution power (Rp) (Gilbert *et al.* 1999), Marker Index (MI) (Prevost and Wilkinson 1999), and Polymorphic Information Content (PIC) (De Rick *et al.* 2001) were calculated for each primer.

Analysis of population structure: Both the phenotyping and genotyping data were used for analysis of the population structure of the procured accessions. Each primer binding site was considered as a locus, and data were scored as presence (1) and absence (0). STRUCTURE software with 10000 burn-in and 100000 MCMC replication with assumed K value; 2–10 and 5 iterations for each number of K value were used for determination of population structure (Pritchard *et al.* 2001).

The optimum number of clusters was estimated through both hierarchical and non-hierarchical approaches using polymorphic ISSR markers data with R-program-based packages. In the hierarchical approach, Unweighted-Pair Group Method Arithmetic Average (UPGMA) clustering was done with calculated Jaccard's similarity coefficient using NTSYSpc (Rohlf 2000). The Non-hierarchical K-mean clustering algorithm was used to identify the optimum number of clusters with the sum of square function of the Elbow method (Syakur *et al.* 2018). Principal component analysis was done for estimating the contribution and correlation among quantitative variables and individuals using R stat packages. The result of PCA was observed as biplot analysis for the individuals and variables.

Estimation of genetic variability: The number of subpopulations was identified by STRUCTURE analysis with the optimum K value. The STRUCTURE output with Q1 and Q2 inferred ancestry of each individual was used for the estimation of genetic diversity analysis (Supplementary Table 3). Genetic diversity was estimated between subpopulations Q1 and Q2 for the gene frequency, analysis of molecular variance (AMOVA), and Principal coordinate analysis (PCoA) using GenAlex software (Pagnotta 2018).

RESULTS AND DISCUSSION

The crop genetic diversity is the demand of plant researchers for identification of novel donors with wider adaptability and high yield potential, having novel genes and alleles that can be utilized with biotechnological approaches (Kumari *et al.* 2021b, Kumar *et al.* 2022, Kothari *et al.* 2024). The widening of the genetic base is the need for breeding climate-resilient cultivars (Rahman *et al.* 2017, Wang *et al.* 2018). The present study was conducted with 22 wild rice accessions of seven different states belonging to six different agro-climatic zones of India (Supplementary Table 1). The choice of a molecular marker is highly crucial to identify the level of polymorphism among them and to discriminate between two genotypes. A dominant marker is the preferred choice for diversity analysis (Nelson and Anderson 2013). It does not require sequence information to design and provide unbiased comparisons of multiple loci of the genome. Among dominant markers, ISSR is most preferable (El-Bakatoushi *et al.* 2018). It has been used for diversity analysis, germplasm characterization, and population identification in various species due to their multilocus and multiallelic characteristics (Tu Anh *et al.* 2018). A total of 15 ISSR primers based on previous reports were chosen for the present analysis. The PIC, MI,

Table 1 Polymorphic information of studied markers among wild rice accessions

S.no	ISSR PRIMERS	5'-3' Sequence	Tm (°C)	MW (bp)	TB	PB	PP	PIC	MI	Rp
1	PRIMER 1	(CT)8G	43	250-1000	5	4	80	0.20	0.80	1.27
2	PRIMER 2	(CT)8A	43	350-1000	3	3	100	0.34	1.01	1.55
3	PRIMER 3	(AC)8G	45	350-1000	4	3	75	0.29	0.87	2.09
4	PRIMER 4	(TC)8A	43	600-1000	3	2	66.6	0.30	0.60	1.45
5	ISSR13	(GACA)4	43	350-750	4	3	75	0.31	0.93	1.91
6	UBC807	(AG)8T	43	300-900	6	6	100	0.37	2.20	3.18
7	UBC808	(AG)8C	45	300-700	4	3	75	0.20	0.60	1.00
8	UBC810	(GA)8T	45	350-1000	4	4	100	0.37	1.48	2.36
9	UBC812	(GA)8A	43	500-1000	4	4	100	0.44	1.76	2.73
10	UBC818	(CA)8G	43	500-600	2	1	50	0.23	0.23	0.73
11	UBC841	(GA)8CTC	48	400-1000	4	4	100	0.46	1.85	3.09
12	UBC842	(GA)8CTG	43	350-900	5	5	100	0.27	1.33	1.91
13	UBC848	(CA)8AAGG	51	350-500	3	3	100	0.14	0.42	0.45
14	UBC857	(AC)8CTG	51	250-1250	5	4	80	0.33	1.33	2.36
15	UBC866	(CTC)6	52	250-1000	5	4	80	0.37	1.48	3.09
Average					4	3.5	85.4	0.3	1.12	1.94
Total					61	53				

MW, Molecular weight; TB, Total band; PB, Polymorphic band; PP, Polymorphic percentage; PIC, Polymorphic information content; MI, Marker index, Rp, Resolving power.

and Rp parameters were calculated to identify the most informative ISSR primer for diversity analysis in rice. It was observed that all the chosen sets of markers had PIC value >0.1 with average PIC value >0.3 (Table 1). It indicated that they were highly polymorphic as per the PIC value for dominant markers (Serrote *et al.* 2020). UBC807, UBC812, and UBC841 were highly informative ISSR primers for rice based on PIC value with high MI and Rp of markers (Table 1) (Serrote *et al.* 2020). The earlier reports identified UBC810 (Terzopoulos and Bebeli 2008) and UBC848 (Salazar-laureles *et al.* 2015) ISSR markers for fababean. UBC890 (Kumar *et al.* 2012), UBC879 (Gautam *et al.* 2016), and UBC818 (Nath *et al.* 2017) as highly informative ISSR markers for sesame, chickpea, and green gram, respectively. For rice, UBC807 has been reported as a highly informative marker for diversity analysis in southeastern and South African countries (Moonsap *et al.* 2019). UBC841 has been reported as a highly informative ISSR marker for diversity analysis in Indian rice (Dharmaraj *et al.* 2018). UBC812 has been reported for medicinal shrubs as a highly informative marker (Alansi *et al.* 2016). The average polymorphic percentage for pulses was reported in the range of 65% (Black gram), 68% (chickpea) to 79% (green gram) for ISSR markers (Pakseresht *et al.* 2013, Das *et al.* 2014, Nath *et al.* 2017). In wheat, 84.8% polymorphism was reported for ISSR (Abou-Dief *et al.* 2013). Similarly, in rice, 71% polymorphism was reported for African rice (Eltaher *et al.* 2018), while 82.96% for rice from south-eastern countries (Moonsap *et al.* 2019). A significantly higher level of polymorphism percentage, 85.4% was observed for the present rice germplasm collected from six different agro-climatic zones of India with the present set of ISSR markers as compared to the earlier reports.

The identification of a number of subpopulations among the accessions studied was the prerequisite for understanding the genetic diversity and ancestry relationship among

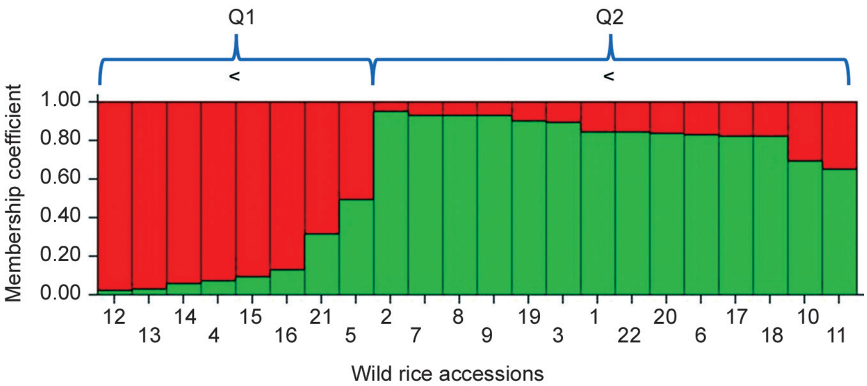

Fig. 1 Determination of population structure using STRUCTURE A) Barplot depicting the distribution of wild rice accessions among two populations; Q1 and Q2. 1, NKSWR214; 2, NKSWR177; 3, NKSWR453; 4, NKSWR402; 5, NKSWR310; 6, NKSWR243; 7, NKSWR190; 8, NKSWR162; 9, NKSWR223; 10, NKSWR117; 11, NKSWR171; 12, NKSWR457; 13, NKSWR372; 14, NKSWR136; 15, NKSWR158; 16, NKSWR110; 17, NKSWR207; 18, NKSWR119; 19, NKSWR168; 20, NKSWR247; 21, NKSWR245 and 22, NKSWR126.

Table 2 Comparison of the identified population for Inferred ancestry, expected heterozygosity, fixation index (Fst), and allelic divergence using STRUCTURE and GenAlex

Features	Populations	
Population types	Q1	Q2
No. of Individuals (N)	8	14
Inferred ancestory	0.402	0.417
Expected heterozygosity	0.417	0.312
Mean Fst (Fixation Index)	0.0081	0.2904
Allele frequency divergence among populations	0.04	
Number of different allele (Na)	1.770	1.672
Number of effective allele (Ne)	1.543	1.415
Shannon Information Index (I)	0.460	0.373
Diversity (H)	0.313	0.248
Unbiased diversity (Hu)	0.357	0.267
Percentage of polymorphism (PP)	80.33%	70.49%

Table 3 Population statistics based on ISSR using AMOVA

Source of variations	Degree of freedom	SS	MS	Est. Var.	Percentage (%) Variance
Among pops	1	28.373	28.373	1.893	17%
Within Pops	20	182.036	9.102	9.102	83%
Total	21	210.409		10.995	100%
Stats	PhiPT		0.172		
	P (rand >= data)		0.001		

SS, Sum of squares; MS, Mean of squares; Est Var, Estimated variance.

them and their subsequent implication for marker-trait association studies (Eltaher *et al.* 2018). Both parametric and nonparametric test was performed to identify the number of subpopulations among them (Alhusain and Hafez 2018). A Bayesian model-based STRUCTURE analysis with optimum K value indicated the presence of two subpopulations (Fig. 1 and Supplementary Fig. 1).

The inferred cluster gave the estimate of the membership coefficient of individuals among a given population, called inferred ancestry, estimated using allelic divergence. The mean value of the inferred cluster of two subpopulations around the center showed the perfect partition of individual accession between populations. The Fst value gave the estimate of the fixation index for alternate alleles in the given populations. It was higher for population Q2, which indicated its high fixation rate. The genetic diversity of Q1 population was higher than Q2 as per the estimate of polymorphic percentage loci, different alleles, effective alleles, Shannon diversity index, and gene diversity (Table 2).

The statistical significance of variance between populations inferred by STRUCTURE analysis was estimated with the partition of molecular variance between populations and within populations. A high variation was observed within a population (83%) as compared to between populations (Table 3).

The significance of variation was estimated with PhiPT value [analogue of fixation index (FST)] that measures population differentiation due to genetic structure (Capo-Chichi *et al.* 2023). The PhiPT value ranged from 0 (no genetic differentiation) to 1 (total genetic differentiation), which was used to estimate the extent to which populations differ in terms of genetic makeup, especially for the dominant markers where information of heterozygous loci is lacking (Mokuolu *et al.* 2024). The PhiPT value was 0.172 (>0.15) with $p<0.001$ indicating large significant differentiation among populations in rice (Frankham *et al.* 2002, Luong *et al.* 2021). The type of subgroup was also analyzed with a non-parametric test using hierarchical (Fig. 2A) and non-hierarchical clustering methods (Fig. 2B and Supplementary Fig. 2). However, two groups were found following STRUCTURE output in hierarchical clustering; AMOVA, PhiPT analysis, and cluster analysis. But non-hierarchical clustering grouped them into four groups that were similar to the findings of principal coordinate analysis (Supplementary Fig. 3) (Syakur *et al.* 2018). Hierarchical and non-hierarchical clustering are the methods of grouping the rice accessions for the study of genetic variation and the development of improved breeding strategies (Sinaga *et al.* 2025). Hierarchical clustering involves an unsupervised model to create clusters in a pre-defined order using top-down and bottom-up approaches to group similar clusters together in a hierarchical manner and develop a tree-like structure (Chhabra and Mohapatra 2022, Yu and Hou 2022). However, non-hierarchical clustering does not follow and develop a tree-like structure; instead, it uses the K-means clustering method, where clusters are formed based on grouping the accession by breaking and merging the clusters. The present study identified two clusters for hierarchical and four for non-hierarchical approaches. The clusters number showed discrepancy in output by both the approaches but closed examination of clustering by Elbow method and PCoA showed that the population Q1 was tri-partite by non-hierarchal clustering methods; Elbow method-based K-mean clustering (Fig. 2B and Supplementary Fig. 2) and PCoA (Supplementary Fig. 3). Here, the highest and lowest values with the average value are used to develop a centroid value and then the distance from the centroid value is used to partition between clusters made in such a way that non-overlapping groups have no hierarchical relationship among them (Oti *et al.* 2021, Ay *et al.* 2023). The non-hierarchical clustering has been used to study the different cultivated and wild rice accessions of *O. rufipogon* and *O. nivara* for their genetic variability (Panda *et al.* 2021, Mutembei and Nyongesa 2024, Singh *et al.* 2024).

The closed examination of clustering by the Elbow method and PCoA showed that the population Q1 was tri-partite by non-hierarchical clustering methods and PCoA. This indicated that both the parametric and non-parametric

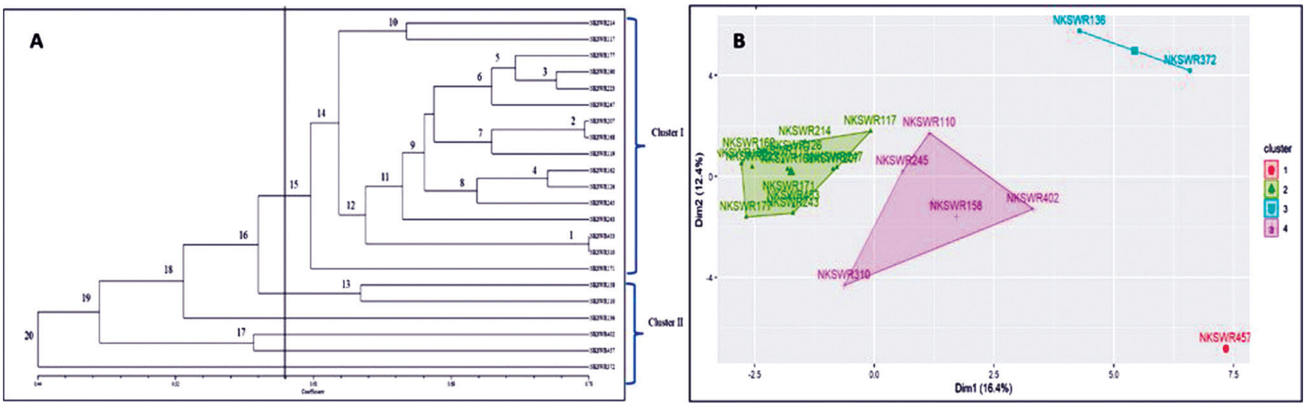

Fig. 2 Clustering of twenty-two wild rice accessions using polymorphic ISSR markers.
A) Hierarchical clustering based on genetic similarity, B) Non-hierarchical based clustering; The cluster plot based on the optimal number of K, the mean clustering method.

analyses were following the STRUCTURE output. Diversity analysis and population structure using highly polymorphic codominant markers such as SSR and SNPs in various studies reported similar population structures for rice in the Asian region (Luong *et al.* 2021).

Assessment of phenotypic variation and genetic diversity in wild rice using molecular markers was the prime objective of the present study, aimed at identifying the ideal genotype with excellent yield contributing traits. ANOVA of wild rice accessions showed a significant variation between them for different morphological traits (Supplementary Table 4 and Supplementary Table 5). Principal component biplot analysis (PCBA) was done to identify the phenotypic traits with higher variance, the correlation of phenotypic variance, and their relationship with wild rice accession (Fig. 3A and B). The amount of variance was measured in terms of eigenvalue. The first three principal components with eigenvalues greater than 1 were taken, which had phenotypic variance with a cumulative percentage of 82.25% (Supplementary Fig. 4 and Supplementary Table 6) (Kaiser 1961). An eigenvalue greater than 1 with a phenotypic variance of more than 65% was reported for germplasm characterization for agronomically important traits in rice (Burman *et al.* 2021). The PCBA showed that PC1 was highly contributed by PN, TN, BY, and SY whereas PC2 was contributed by PH, DTF, and PL (Fig. 3A). The present study showed that PN, TN, and BY were major contributing traits for discriminating between wild rice accessions. TN and PN were highly correlated traits for yield contribution. SY was found to be highly correlated with BY, PN, TN, PL, and PH (Mvuyekure *et al.* 2018). The relationship of agronomically important traits with germplasm accessions was also analyzed with biplot analysis (Fig. 3B).

PCBA revealed that germplasm accessions NKSWR 372, NKSWR 457, NKSWR 126, and NKSWR 145 might be used as donors for seed yield in crop improvement programmes (Mvuyekure *et al.* 2018). Seed yield in NKSWR 372 was contributed by higher TN, PN, and lower PH (Fig. 3B), whereas seed yield in NKSWR 457 was contributed by high PL. Seed yield in NKSWR 245 was contributed by PL, PN, and TN (Fig. 3B). Early flowering was observed in NKSWR 402, NKSWR 158, NKSWR 168, NKSWR 247, and NKSWR 171. Thus, these genotypes may be used as the donor for developing

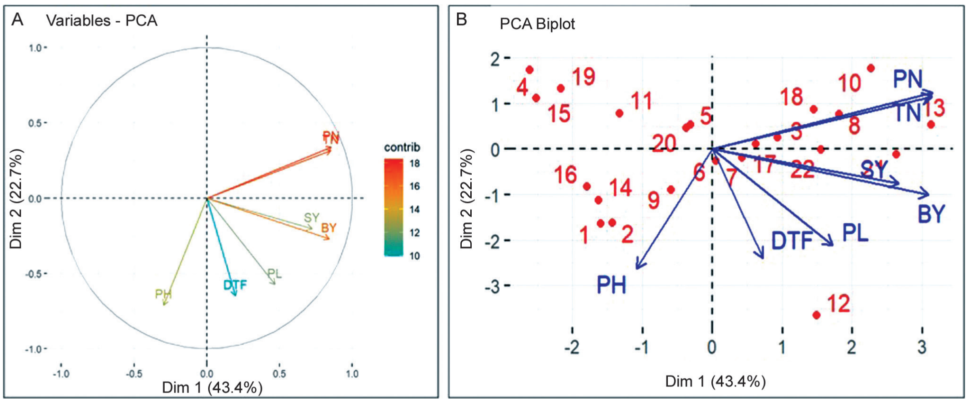

Fig. 3 PCA analysis for yield contributing variables and presentation of the quality and the contribution of variables to PC1 and PC2 using biplot analysis. A) Biplot analysis for the visualization of agronomically important trait for their correlations and quality contribution to phenotypic variation as PC1 and PC2; B) PCA Biplot analysis for the different trait and genotype for PC1 and PC2. (PN, Panicle number; TN, Tiller number; PL, Panicle length; SY, Seed yield per plant; BY, Biological yield per plant; PH, Plant height; DTF, Days to fifty percent flowering). 1, NKSWR214; 2, NKSWR177; 3, NKSWR453; 4, NKSWR402; 5, NKSWR310; 6, NKSWR243; 7, NKSWR190; 8, NKSWR162; 9, NKSWR223; 10, NKSWR117; 11, NKSWR171; 12, NKSWR457; 13, NKSWR372; 14, NKSWR136; 15, NKSWR158; 16, NKSWR110; 17, NKSWR207; 18, NKSWR119; 19, NKSWR168; 20, NKSWR247; 21, NKSWR245 and 22, NKSWR126.

short-duration rice varieties.

From the present investigation, we can conclude that ISSR markers provide an optimum polymorphism for diversity analysis. With 15 markers only, 53 polymorphic loci were identified, which indicated that these are cheaper and multilocus, and it emphasizes their role in unbiased comparison of germplasm for diversity analysis. Two sub-populations were observed based on parametric and nonparametric approaches that may be useful for marker-trait association studies. PCBA identified NKSWR 372, NKSWR 457, NKSWR 126, and NKSWR 145 as potential donors for yield in various crop improvement programmes of rice.

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