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Exploring the underlying genetic polymorphism in wheat genotypes employing ISSR markers

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Abstract
A total of 37 alleles were amplified using five ISSR primers, with an average of 7.4 polymorphic bands per primer. The number of alleles per locus ranged from 5 to 12, while the polymorphism information content (PIC) values varied between 0.675 and 0.868. ISSR primers demonstrated strong performance in detecting genetic diversity in wheat and are recommended for future wheat improvement programs. Genomic DNA was extracted from 33 wheat genotypes using the CTAB method and subsequently amplified with the ISSR primers. The similarity matrix of the genotypes was calculated using NTSYSpc 2.1 software, and clustering was performed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method. The primary objective of this study was to assess genetic polymorphism and diversity, select promising diverse progenitors, and identify putative and informative markers that could be valuable for future crop improvement initiatives.

Keywords: Simple sequence repeat, bread wheat, genetic diversity, markers, polymorphism, similarity coefficient, cluster

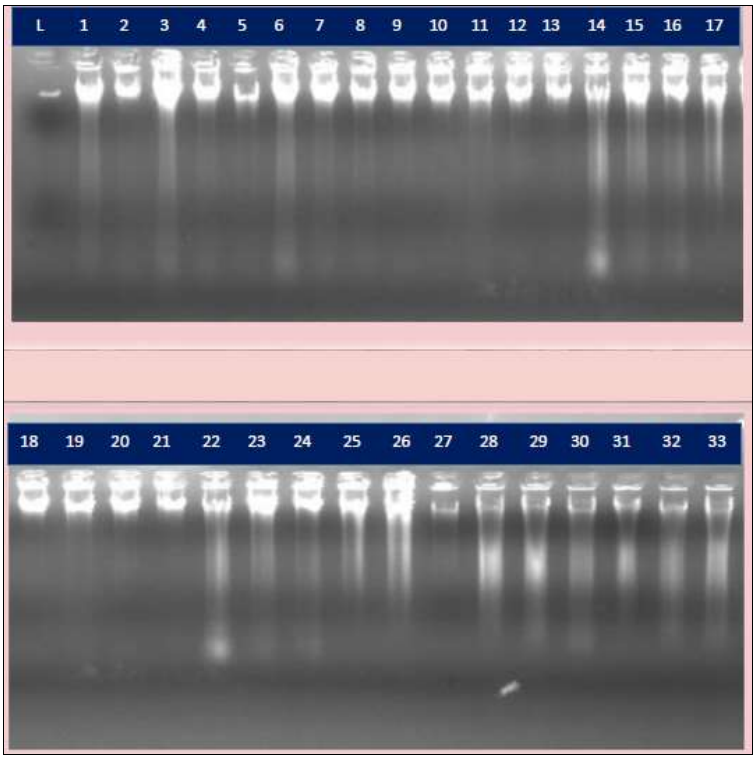
Introduction
Wheat is the foremost and strategic cereal crop of the world and major staple food of more than thirty six percent of the world`s population. Common bread wheat (*Triticum aestivum* L.) is a allohexaploid (2n=6x=42, AABBDD genome) with three homeologous genomes, self-pollinating, annual crop plant, C3 plant, monocot, long day and cleistogamous plant. It belongs to family Poaceae, and it is the most diverse, largest, complex genomes of cereals. Knowledge of genetic diversity is essential for understanding the relationships between cultivars, facilitating their classification and characterization.
Molecular markers is a new frontiers approaches for enhancing efficiency in selection of highly diverse for progenitor and effective use of genetic resources in crop improvement. The tremendous types molecular markers have been used for wheat effective Genotyping [1] like, Restriction Fragment Length Polymorphism (RFLP), Randomly Amplified Polymorphism DNA (RAPD), Single Nucleotide Polymorphism (SNP), Simple Sequence Repeat (SSRs) and Inter Simple Sequence Repeat (SSRs) markers. Inter Simple Sequence Repeat (ISSRs) markers determined by the means of repeat sequences, anchored primers that are amplified simple sequence repeats, and has also been applied for assess the genetic polymorphism in crop species. This technology is the highly informative and putative due to high repeatability and polymorphism and also efficient tools for detection of genetic diversity in plant species [3, 4].
The prime objective of this manuscripts were to 1) Determination of the genetic genetic diversity and genetic relationship and identify putative and informative markers for genetic enhancement of crop.

Material and Methods
A. Plant materials
The Thirty three wheat genotypes obtained from the Agricultural Research Institute, Niphad, Nashik, Maharashtra. All wheat seeds were sown in small clay potted in a growth chamber providing normal growing conditions.

After about three weeks seeding has grown 3-4 seedlings were cut and packed in plastic bags and stored at – 80 °C for DNA extraction.

Table 1: List of wheat genotypes used for analysis

Sr. No.	Name of Genotypes	Pedigree
1.	NIAW-2778	NIAW 1188/NIAW 1161
2.	NIAW-2976	NIAW-1121/NIAW-2844
3.	NIAW-2822	PBW 343 / LOK 54
4.	NIAW-2823	NIAW 1161/NIAW 1188
5.	NIAW-2930	POTCH93/4/MILAN/KAUZ//PRINIA/3 /BAV92/5/MILAN/KAUZ//PRINIA /3/BAV92
6.	NIAW-2757	NIAW 917/MP 3097
7.	NIAW-2844	NIAW 34/LOK 54
8.	NIAW-2874	PHS 623/NIAW 1121
9.	NIAW-2892	WR 1392/NIAW 1161
10.	NIAW-2959	CNO79//PF70354/MUS/3/PASTOR/4/BAV92/S/FRET2/KUKUNA//….
11.	NIAW-2721	NIAW 301/HD 2781
12.	NIAW-2725	NIAW 301/FLW 6
13.	NIAW-2792	NIAW 1188/NIAW 1161
14.	NIAW-2809	PBW 343/WH147
15.	NIAW-2837	LOK 45/RAJ 4083
16.	NIAW-2891	LOK 54/VW 0514
17.	NI-5439	RFPM 80/NP7103
18.	NIAW-1415	GW 9506/PRL//PRL
19.	NIAW-1885	ALTAR84/AEGILOPS QUARROSSA (TAUS)// OPATA/3/...
20.	NIAW-2565	MILAN/MUNIA/3/PASTOR//MUNIA/ALTAR8414/MILAN/DUCULA
21.	NIAW-1994	NIAW 34/PBW 435
22.	NIAW-34	CNO79/PRL'S'
23.	MACS-6222	HD 2189*2//MACS 2496
24.	HD-2189	HD1963/HD1931
25.	HD-2932	KAUZ/STAR/HD2643
26.	NIAW-2495	NIAW 1161/NIAW 1188
27.	NIAW-2539	PRU/ 2*PASTOR/ISUNSTATE
28.	NIAW-2547	BAV92//IRENA/KAUZ/3/HUITES/4/DOLL
29.	NIAW-2613	PFAU/SERI.1B//AMAD/3/INQALAB91*2/KUKUNA/4/WBLL1*2/KURUKU
30.	NIAW-2030	Lok 45 X NIAW 34
31.	NIAW-2595	PRET2*2/4/SNI/TRAP#1/3/KAUZ*2/TRAP//KAUZ*2/5/BOW/URES//2*WEAVER/3/CROC-1/AE. SQUARROSA (213)// PGO
32.	NIAW-917	GW 244 / Bob White
33.	NIAW-301	SERI 82/3/MRS/JUP/HORK 'S'



Plat 1: Quality of genomic DNA of wheat genotypes

B. DNA Extraction

Genomic DNA was extracted from thirty three wheat genotypes using CTAB procedure reported by[10]. Leaves of six 3-week-old plants grown from representative seeds of any accession. To this end the young seedling leaves of each accession were harvested and frozen in liquid nitrogen. Subsequently, approximately 3-5 g of leaf material was ground into a fine powder and poured DNA extraction was performed according to the modified CTAB-method. The quantity of DNA was measured with a UV spectrophotometer at 260 nm and adjusted to a concentration of 100 ng/ml. Amplification products were separated by electrophoresis (60V, 60 ma, 100W, 60–90 min) in 0.7% agarose gels in 1 TAE buffer stained with 2 ml/100 ml ethidium bromide. The gel was scanned with a transilluminator and amplification into 50 ml propylene tubes.

C. Polymerase chain reaction (PCR) amplification

In this study, all 8 SSR primers were used for marker analysis. PCR conditions were maintained as described [11]. Each PCR was carried out in a 20-µL reaction volume, containing 14.1 µL double-distilled deionized H2O, 2.0 µL 10X buffer wth 15Mm $Mgcl_2$, 1.6 µL dNTPs, 0.3 µL Taq polymerase, 1 µL of primer, and 1 µL DNA.

The thermal cycling consisted of 5 min at 94 °C (initial denaturation); 40 cycles of 1 min at 94 °C, 1 min at the annealing temperature (50, 55, or 60 °C), and 1 min at 72 °C; followed by a final extension at 72 °C for 10 min. PCR amplification products were separated by electrophoresis on 3% TBE agarose gels at 80 Voltage for 1 hr and stained with ethidium bromide for visualization under UV transilluminator.

Table 2: Sequenced and fixed optimum annealing temperature for SSR primers

Sr. No.	Primers	Sequenced of Primers (5`-3`)	Ta
1.	ISSR-801	5`AGC AGC AGC AGC AT 3`	46 °C
2.	ISSR-803	5`CAC ACA CAC ACA AC 3`	38 °C
3.	ISSR-804	5`CAC AGA CAC ACA GT 3`	40 °C
4.	ISSR-810	5`GTG TGT GTG TGT CA 3`	40 °C
5.	ISSR-812	5`GTG TGT GTG TGT AT 3`	36 °C

Data analysis

Each band amplified by each primer was scored as present (1) or absent (0) for the thirty three cultivars, and the data were entered into a binary matrix as discrete variables (1 for presence and 0 for absence of a homologous fragment). The gene diversity also called polymorphic information content (PIC) was computed according to [12] as:

$$PIC = 1 - \sum_{i=1}^{n} Pi^2$$

Where, n is the number of band positions analyzed in the set of accessions and Pi is the frequency of it pattern. Matrix similarity of genotypes was calculated by using NTSYSpc.2.1 with Sanh-clustering using the UPGMA (Unweighted Paired Group Method Using Arithmetic Averages) method [13, 14]. We used two different coefficients: Band and Dice .The results are presented graphically in dendrograms.

Results and Discussion

To assess genetic distance among 33 wheat genotypes. A total of 37 fragments was obtained from the 5 SSR primers and all the bands were polymorphic across all the genotypes screened. 37 of them were polymorphic, from which 6 were a unique band. The number of alleles ranged from 5 to 12. (Table 9).

Table 9: Information contents of ISSR primers used for wheat divergence analysis.

Sr. No.	Primer	No. of bands amplified	Poly-morphic bands	Mono-morphic bands	Unique bands*	%Poly- morphic bands	Size range (bp)	PIC
1.	ISSR-801	12	06	06	01	50	140-1200	0.867
2.	ISSR-803	05	03	02	00	60	402-805	0.790
3.	ISSR-804	10	10	00	02	100	205-700	0.793
4.	ISSR-810	08	08	00	02	100	230-825	0.675
5.	ISSR-812	10	10	00	01	100	240-900	0.868

*Unique bands are also counted under polymorphic

The average number of alleles/locus was 7.4 and the maximum number of scorable alleles was detected at locus ISSR-801(12 alleles) and minimum number of alleles detected in ISSR-803 primer (5 alleles) (Table 2).

The all primers showed 88% polymorphism. Maximum number bands reported in NIAW-2809 and NIAW-2547(30 loci) and minimum number bands observed in NIAW-2792(16 loci) cultivar of wheat. The present study shows a relatively high level of polymorphism (88%) among the 33 wheat genotypes. The ISSR analysis produced size of amplification products ranges 140 bp-1200 bp. In this study, the polymorphic information content values per locus ranged from 0.67 for the ISSR-810 locus to 0.868 for ISSR-812 with an average of 7.4 for all loci (Table 1). The all primers showed PIC values 0.5 or more indicating that these primers may be considered as more informative.

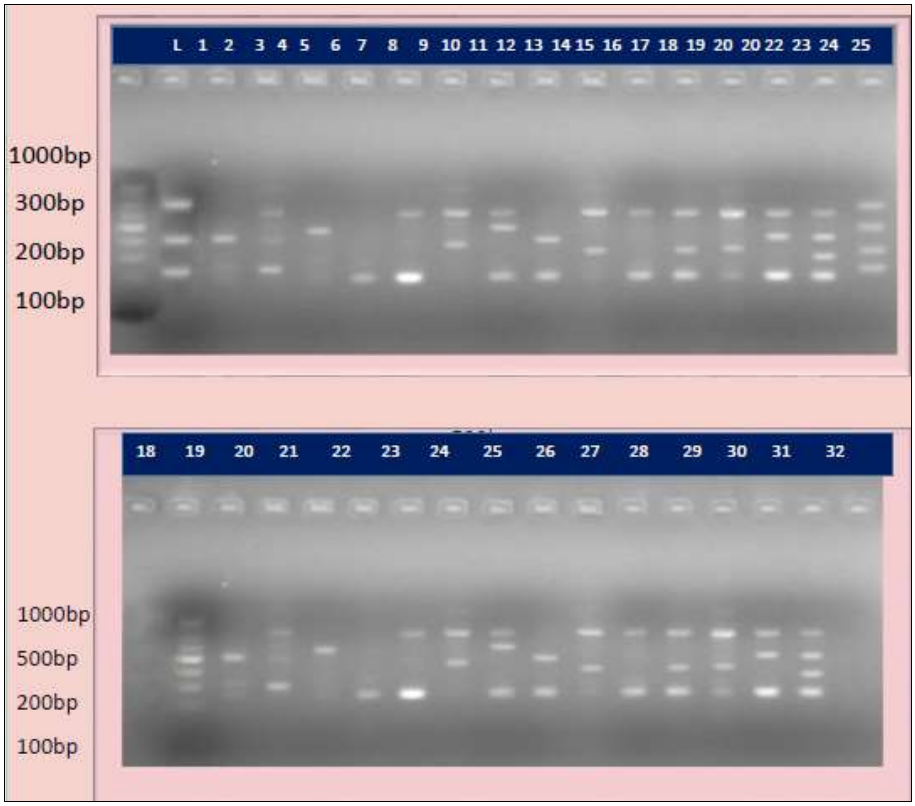


Plate 2: DNA amplification by ISSR-804

Phylogenetic Data

The diversity observed in the thirty three wheat genotypes for mainly attributed to the genetic dissimilarities. The Dice similarity coefficient values among 33 wheat genotypes investigated is presented in Table 11. The pairwise similarity and genetic distance values ranged from 0.44 to 1.00. The maximum Dice similarity coefficient value 1.00 was present between NIAW-2595 genotypes indicating that there is less genetic variability among these genotypes and the minimum similarity coefficient value of 0.44 was observed among NIAW-2891 indicating that the genotypes are being the most differentiated and divergent of the thirty three genotypes. Those cultivars that display similar coefficients of similarity are genetically close to one another, while those having variable coefficients of similarity are dissimilar.

To visualize the genetic relationship among 33 wheat genotypes, a dendrogram was constructed based on the UPGMA method from similarity matrix using NTSYSpc 2.02i Programme was presented in (Fig. 2). Based on cluster analysis using ISSR markers, genotypes were grouped into two major clusters (I and II). First major cluster I consisted of four sub clusters (Ia, Ib, Ic and Id). First sub cluster Ia comprised two genotypes *viz.*, NIAW-2778 and NIAW-1415. The second sub cluster (Ib) further divided into six sub subcluster Ib (i), Ib (ii), Ib (iii), Ib (iv), Ib (v) and Ib (vi). First sub subcluster Ib (i) consists one genotype *viz.* NIAW-2822 and formed independent sub subcluster. Second sub subcluster Ib (ii) comprised seven genotypes *viz.*, NIAW-2930, NIAW-2892, NI-5439, NIAW - 2721, NIAW-2809, NIAW-2837 and NIAW-2725. Third sub subcluster Ib (iii) consists five genotypes *viz.*, HD-2932, NIAW-2539, NIAW-2547, NIAW-2495 and NIAW-2613. Fourth sub subcluster Ib (iv) comprised four genotypes *viz.*, NIAW-2565, MACS-6222, NIAW-1994 and HD-2189. Fifth sub subcluster Ib (v) consists three genotypes *viz.*, NIAW 2823, NIAW-2874 and NIAW-2959. Six sub subcluster Ib (vi) consists one genotypes *viz.*, NIAW-34. Third sub cluster Ic comprised one genotypes *viz.*, NIAW-2844 and formed independent sub cluster. Fourth sub cluster Id comprised five genotypes *viz.*, NIAW-2976, NIAW-1885, NIAW-2757, NIAW-2792 and NIAW2030. Second major cluster II consisted of two sub clusters (IIa and IIb). First sub cluster (IIa) comprised three genotypes *viz.*, NIAW-2595, NIAW-301 and NIAW-917. Second sub cluster (IIb) consists of independent sub cluster and comprised only one genotypes *viz.*, NIAW-2891.

In the present investigation out of 8 primers used 5 primers were able to amplify the genomic DNA of wheat and 3 primer were not able to amplify the genomic DNA. The total of 45 bands was resolved by 5 ISSR primers out of which 37 bands were polymorphic and 8 bands monomorphic. The PIC values of primers ranged from 0.675 to 0.868. Further, it was observed that there was no correlation between per cent polymorphism and PIC values as ISSR primers 810 showed minimum PIC values but was 100 per cent polymorphic.

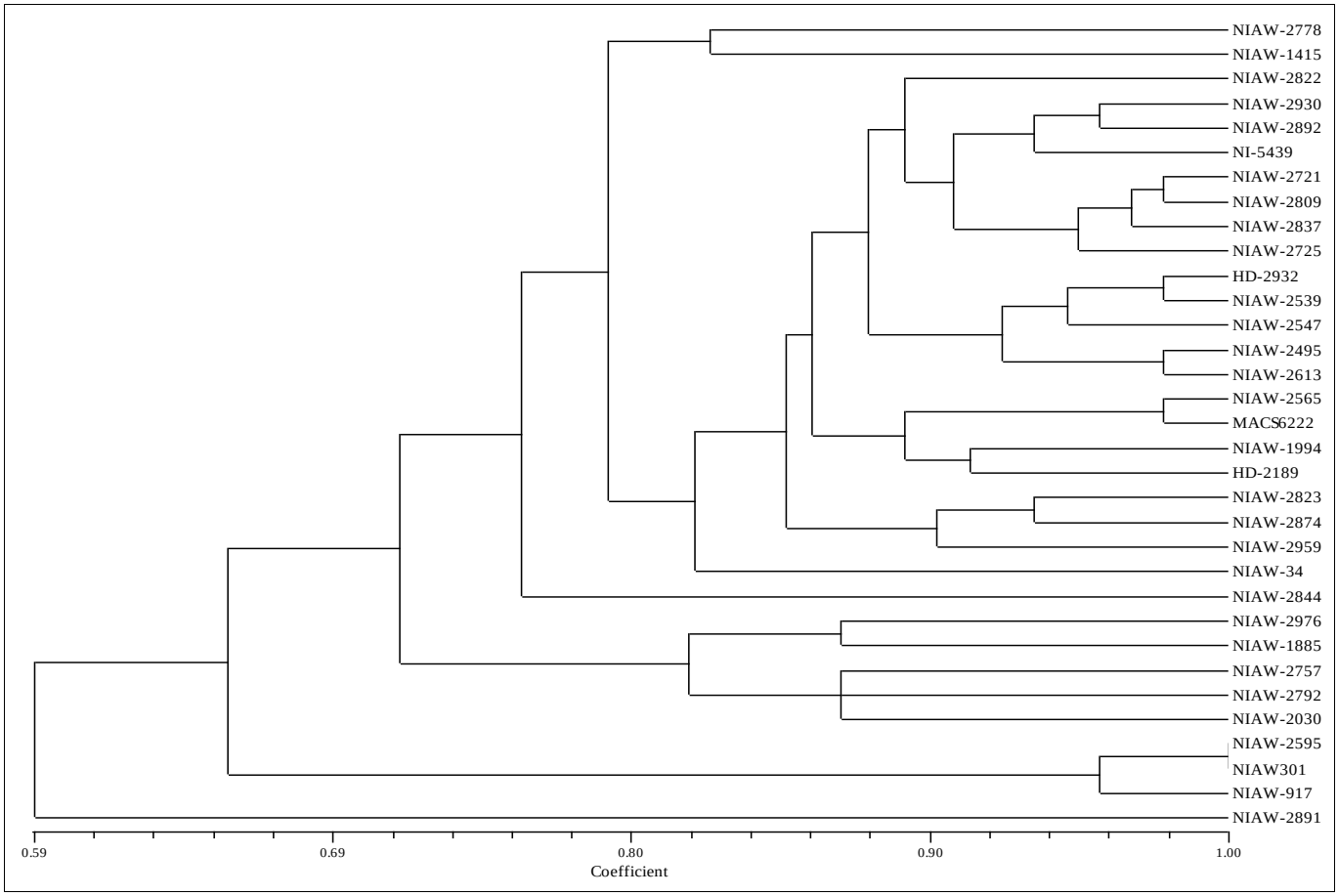


Fig 1: Consensus tree showing clustering of thirty three wheat genotypes using ISSR markers.

In the present study, Dashchi *et al*. (2012) [22] out of 57 ISSR primers screened, 23 anchored dinucleotide primers amplified 267 clear and discernible loci which 224 were polymorphic (83.9%). The frequency of all amplified loci was more than 5%. No less common loci with frequency lower than 50% were amplified. The number of total loci per primer ranged from 7 (UBC844) to 16 (UBC811), with an average of 11.6. The number of polymorphic loci per primer ranged from 5 to 14 with a mean value of 9.78. The size of the amplified fragments ranged from 75 to 2000 bp. The Dice similarity coefficient for the germplasm ranged from 0.76 to 0.91 .The genotypes in different groups might be used as potential parents in bread wheat breeding programs.

Abou-Deif *et al.* (2013) [23] observed eight ISSR primers produced 112 amplified DNA fragments ranging in size from 127–1857 base pairs, 17 fragments were monomorphic (15.2%) and 95 fragments were polymorphic (84.8%) with average of 11.87 polymorphisms per primer. Similarity values showed clearly substantial differences among the wheat varieties, with genetic similarity ranging from 47 to 94%, with an average of 71%. The 112 markers were used to construct the dendrogram based on UPGMA cluster analysis. The dendrogram indicated that the ISSR markers succeeded in distinguishing most of the 20 varieties in relation to their genetic background and geographical origin.

In a study investigated to evaluate the genetic diversity among 25 genotypes of durum wheat through ten ISSR markers. The average PIC was 0.41, and the highest amount PIC was 0.49 related to primer IS12. The highest genetic distance between genotypes based on Dice similarity coefficients was observed between genotypes 23 and 24 and the least genetic distance was between genotypes 13 and 18. The results revealed that ISSR markers could be efficiently used to evaluate genetic variation in the durum wheat genotypes wheat germplasm and could be used in isolating gene (s) which controlling some important traits**.**

Conclusion

Our result illustrated that the common bread wheat genotypes harbor huge amount of genetic diversity. Broadening of genetic diversity depicted a clear insight effective parent selection in plant breeding. ISSR markers is the most promisin tools that hold great potential in crop improvement.

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