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Comparative suitability of phenotypic expressions and microsatellite markers in classifying oat genotypes (*Avena sativa* L.)

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Abstract
A comparison was made between phenotypic and microsatellite markers (ISSR) to assess the suitability of the two marker systems in classifying oat genotypes representing different agroecological zones. An average range of genetic similarity (0.84-0.20) was observed on the basis of 20 ISSR markers whereas, it was found high (0.995-0.204) on the basis of 7 primary rainfed morphological expression. A random grouping was observed in dendrogram based on the ISSR markers, while dendrogram based on phenotypic character clustered genotypes into their respective geographic groups. A negative correlation (r = -0.186) was found among morphological and molecular marker systems, but the latter could be an effective tool in distinguishing the genotypes using specific band positions for them. The genotypic classification agreed closely with the grouping observed in ISSR based 3D analysis and the association technique came with best discrimination among all genotypes.

Keywords: ISSR marker, Oat, Phenotypic expressions, Relationship

Introduction

Oat (*Avena sativa* L.) is one of the most important forage and feed crops of the world. Oat is used for green fodder, straw, hay or silage. Oat grain makes a good balanced concentrate in the rations for poultry, cattle, sheep and other animals. Green fodder contains about 10-13% protein and 30-35% dry matter (Mishra and Verma, 1985). Despite being high fed fodder crop, it is now gaining importance due to its unique and important quality characteristics, particularly lipid and protein in grains (Ruwali and Verma, 2013). Hence, the first and foremost need is the identification or cataloguing of oat genotypes along with the assessment of genetic diversity prevalent in different geographical regions in the world. The genetic diversity analysis in oats has been done using either morphological characteristics or molecular markers such as RAPD (Loskutov and Perchuk, 2000), SSR, RFLP (Pal, 2002) SCAR and CAPS (Molnar and Orr, 2008). But there are meager reports on the identification and characterization of oats germplasm using both the morphological and molecular markers so as to compare the two systems. However, the use of qualitative and quantitative morphological characteristics is often affected due to genotype x environment interaction, hence not much dependable but still they are easy to compare the phenotypic status of the plant with the genotypic level.

Among a large category of molecular markers, microsatellite markers (ISSR) can be efficiently applied to identify useful polymorphisms (Rafalski and Tingey, 1993; Doldi *et al*. 1997). The resolving power of this tool is several folds higher than morphological or isozyme markers and is much simpler and technically less demanding than RFLP and other new generation markers. Molecular markers have proved their importance for diversity analysis in several crops and horticultural plants like neem (Deshwal *et al.,* 2005), common bean (Marotti *et al*. 2007), strawberry (Kuras *et al.,* 2004) and in oats (Wight *et al.,* 2003). Since molecular based characterization of genotypes is independent of G × E interaction it may be an efficient and effective tool to understand and explain the genotypic dissimilarity between and within geographical regions and ultimately in granting protection and crop improvement program. A comparison between the phenotypic and molecular markers for estimating genetic relationship may provide more critical assessment. Research information on such aspects are generally available, however meager literature is documented on this topic in oats. The purpose of this study was to compare the suitability of using

phenotypic expression and molecular markers for classifying and finding relationship in certain oat genotypes.

Materials and Methods

The genetically pure seed of 20 high yielding oat genotypes representing their origin from different geographical locations of the world were collected (Table 1).

***Phenotypic expression analysis*:** All the 20 varieties of Oat were planted in the experiment field at the Instructional Dairy Farm, Nagla, G.B. Pant University of Agriculture and Technology, Pantnagar in a plot size of 30.0 x 6.0 m (one meter long 5 rows spaced 20 cm apart for each variety), replicated thrice in randomized complete block design with no irrigation (rainfed condition) during the crop growing period in *rabi* 2010 and 2011 for two consecutive years so that maximum true characters/ QTL's get expressed in the test genotypes. Seven primary quantitative characteristics were recorded to analyze the genotypic variability among the treatments including number of productive tillers counted at 50% flowering, at 100% flowering stage tiller diameter, biological yield, grain yield and straw yield per plant respectively were studied while plant height and days to maturity were studied at physiological maturity.

Data analysis: The mean of two-year data on phenotypic observation was used to calculate the Euclidean dissimilarity matrix to find out the genotypic relationship using NTSYS pc 2.11v software. Dendrogram was constructed using Euclidean distance coefficients and the correlation between the tree and similarity matrices was estimated by means of the Mantel matrix correspondence test (Mantel, 1967).

ISSR analysis: A total of 26 ISSR primers based on dinucleotide repeats with alternate nucleotide end were tested and out of which 20 polymorphic ones were used to genotype test cultivars evaluated under rainfed conditions. Total genomic DNA was extracted using the method of Doyle and Doyle, 1990 from ten days old seedlings. The PCR reactions were carried out in a 25-ìl volume PCR tube. 20-ìl master mix was prepared

Table 1. Oat genotypes with their origin and pedigree

S.No.	Genotypes	Origin	Pedigree
1.	D. Sel.-1	Pantnagar	Derivative of UPO 201/ UPO 211// UPO 212
2.	D. Sel.-5	Pantnagar	Derivative of UPO 211 x UPO 212
3.	D. Sel.-6	Pantnagar	Derivative of UPO 201 x UPO 210
4.	Wright	U.S.A	Introduction from USA
5.	HFO 114	Hisar	Selection line 37/ 14
6.	OL 125	Ludhiana	Derivative of Appler × IPC-163
7.	UPO 265	Pantnagar	Selection from UPO 201 x Kent
8.	UPO 270	Pantnagar	Selection from UPO 228 x UPO 202
9.	UPO 271	Pantnagar	Selection from UPO 212 x UPO 136
10.	UPO 273	Pantnagar	Selection from UPO 202 x UPO 201
11.	UPO 275	Pantnagar	Selection from Wright x UPO 233
12.	Kent	Australia	Introduction fron USA
13.	UPO 212	Pantnagar	US 1492 x Kent
14.	No. 1	Pantnagar	Selection from local material
15.	OS 6	Hisar	Derivative of HFO 10 x HFO 55
16.	EC 605833	Exotic	Exotic material
17.	EC 605836	Exotic	Exotic material
18.	EC 605838	Exotic	Exotic material
19.	UPO 260	Pantnagar	Derivative of UPO 204 x UPO 211
20.	EC 246199	Exotic	Exotic material

individually for each marker type and DNA amplifications were performed in PTC-100 thermocycler (MJ Research Thermocycler) with 30 cycles of 60 s at 94°C, 60 s at 51°C and 2 min at 72°C. Samples of 10 ìl PCR products were analyzed on 1.8% agarose gel in 0.5X TBE buffer running at 50 V for 4 h. The gels were stained using ethidium bromide solution. After separation, gels were documented using Gel Doc system (Bio-Rad). The amplified products were scored twice manually and independently for each primer. Only clear polymorphic ISSR bands of various molecular weight sizes were scored manually in binary formula of 1 or 0 for their presence or absence, respectively, mobility were considered as a single locus. The total numbers of bands, polymorphic bands, and average number of bands per primer with polymorphism percentage were calculated. Similarity matrix for ISSR primers was constructed using the Jaccard's similarity coefficient values to find out genotypic relationship. These data were then subjected to UPGMA (unweighted pair-group method with arithmetic averages) analysis to generate dendrogram using NTSYS pc-version 2.11v. Principal coordinate (3D) analysis was performed in order to highlight the resolving power of the ordination.

Results and Discussion

***Genetic relationship as revealed by phenotypic expression*:** On an average 85% genetic similarity was observed among 20 genotypes on the basis of Euclidian dissimilarity matrix. The dendrogram based on the Euclidean distance coefficient clustered 14 genotypes in the major cluster and 6 genotypes in the minor cluster. All genotypes from exotic collection included in the study *viz*., EC 246199, EC 605836, EC 605838 and EC 605833 fell into a single cluster breaking at 0.15 dissimilarity coefficient value. Similarly, four genotypes from Pantnagar *viz*., UPO 273, UPO 270, UPO 265 and D.Sel.-6 fell in same cluster breaking at 0.09 dissimilarity coefficient value. In agreement to the clustering pattern from molecular marker data, the clustering from rainfed plot data also placed the genotype HFO 114 and D.Sel.-5 close to each other showing more than 99% similarity between them (dissimilarity coefficient value 0.006). The maximum dissimilar pair identified from the rainfed dissimilarity coefficient was between UPO 260 and EC 246199 (>79%). In PCA analysis, total 8 components separated each genotype from each other in the 3D graph explaining for 100 percent variation.

***Genetic relationship as revealed by ISSR marker*:** The Jaccard's similarity coefficient estimates between pairs of different genotypes included in the study (Table 3) indicated a range of genetic similarity values, which varied from 0.20 (*i.e.* 20%) [between UPO 270 and EC 605836] to 0.84 (*i.e.* 84%) [between HFO 114 and D. Sel.-5]. Among the 20 genotypes the three pairs with lowest GS value *i.e.* maximum diverse pairs were EC 605836 and UPO 270 (20% genetic similarity), KENT and UPO 212 (21% genetic similarity), UPO 212 and EC 246199 and UPO 260 and EC 246199 (with GS value 24% respectively). Genetic similarity between D. Sel. series genotypes varied from 69-75% showing high similarity between them. The test genotypes were also being differentiable based on the absence or presence of amplified bands with different primers. *e.g.* D.Sel.-1 can be differentiated from Wright based on the amplification pattern obtained with primer 2 at 2000 bp and with primer 14 at 1400 bp.

Thus, combination of amplification pattern obtained with two primers can be used effectively to distinguish different pairs of test genotypes. Table 2 contains the total number of ISSR loci generated by each primer and number of polymorphic loci and other details for each primer. Amplified fragments varied in size from 400 bp to 2000 bp.

Table 2. Summary of ISSR amplified products in 20 genotypes of oat

Specifications	Particulars
Total number of primers tested	26
Number of polymorphic primers	20
Total number of monomorphic primers	6
Total number of unique bands identified	1
Total number of bands amplified	94
Size range of amplified products (in bp)	400 to 2000
Average number of bands per primer	4.7
Total number of unique bands identified	01
Total number of polymorphic bands identified	75
Total number of monomorphic bands identified	19
Percentage of all bands that were polymorphic	79.78%

The dendrogram constructed from ISSR marker analysis in oats revealed that the first cluster broke at 0.31 Jaccard's coefficient of similarity, which separated UPO 212 from all other genotypes (Fig. 1). The major gene cluster consisted of 19 oat genotypes leaving one genotype in the first minor gene cluster *viz*., UPO 212. Within the major gene cluster D.Sel.-5 and HFO 114 were not further separated indicating the high level of genetic similarity (>84%) between the two, i.e. some ancestral relationship between them.

The secondary gene cluster was formed within the major gene cluster at 0.325 Jaccard's coefficient of similarity. Thus UPO 212 and EC 605836 separated distinct from rest of the genotypes while the tertiary cluster broke at 0.385 Jaccard's coefficient value where major gene cluster comprised of 14 genotypes while the four genotypes *viz*., UPO 260, EC 605833, OS 6 and Wright fell in the minor gene cluster.

In the present study twenty-six ISSR primers were used; twenty of them showed polymorphism (50-100%) and clustered most genotypes according to their place of origin. Similar kind of findings had also been reported by Loskutov (2007) and Wight *et al.* (2003). The DNA based marker technology imparts a diagnostic tool that permits direct identification of genotypes or strains provided that the DNA marker is closely linked to the trait of interest. In this study a unique band, was identified with primer 14 in UPO 260 of 600 bp, which can be used for identification and characterization of this genotype. The number of loci for each ISSR primer used in the study averaged around 4.7 loci per primer with average polymorphism content of 79.78%; similar detections have been reported by Wei-Tao *et al.* (2009) using ccSSR markers. Deletions, insertions, chromosomal inversion, etc. might be the main causes of differences at the DNA level which generate polymorphism or allelic diversity.

Genotypes EC 605836 and UPO 270 had the lowest genetic similarity value (0.20) revealing that they were the most diverse pair of genotype used in the experimental material thus establishing the utility of microsatellite/ ISSR markers in identifying diverse pairs. In a similar attempt Da-Silva *et al.* (2011) studied diversity among cultivated oat varieties and validated the transferability of genome using microsatellite markers.

***Association between molecular marker and rainfed classification*:** From the similarity and dissimilarity coefficient tables (Table 3 & 4), it can be concluded that the genotype HFO114 and D.Sel.-5 were genetically very similar to each other since the genomic similarity value for them is coming high based on rainfed classification as well as molecular marker (ISSR) data analysis. This mark a possibility that the ISSR markers used in the study may be linked to the genomic region in these genotypes, which governs one or the other observation taken in the rainfed plot. Also, UPO 260 and EC 246199 were identified as the most dissimilar pair in both rainfed data (75%) and ISSR analysis (75%). Similar kind of association was established by using AFLP markers for various traits including plant height and grain yield in oat (Achleitner *et al.,* 2008). Thus, this probable linkage between the ISSR marker and observation taken in rainfed plots needs to be validated through precise investigation for association either through bulk segregation analysis or near isogenic lines (Tanhuanpaa *et al*., 2007). ISSR markers have been used successfully to generate more repeatable microsatellite markers, thus can be used for characterization studies (Lian *et al.,* 2001)

In addition, the relationships between the Euclidean distance matrix based on rainfed plot observations and ISSR markers were analyzed using the matrix correlation approach developed by Mantel (1967). A certain agreement of test for association between the two *i.e.,* ISSR marker variability test and rainfed plot observations came but on negative side, as evidenced by a low and non-significant correlation (r = -0.186) which is further confirmed by a negative non-significant Mantel *t*-test value (*t* = -0.9753) between the morphological genetic distance matrix and the ISSR marker matrix. A weak correlation between Euclidean based genetic distance matrices indicated the discrepancy between the rainfed plot morphological data and ISSR markers based analysis which is also supported by the broad range of genetic similarities (0.204-0.995) based on morphological rainfed expressions as compared to the broad but less similar range based on ISSR (0.20-0.84) analysis.

The lesser dissimilarity (greater similarity) from rainfed plot (Table 4) might be due to similar environmental conditions and hence the near equivalent G × E interaction resulted in similar and narrow pattern of response of genotypes. The two methods were not found comparable in distinguishing all the genotypes individually which was evident by the dendrogram patterns (Fig. 1 & 2) and 3D analysis. Several other comparisons between morphological and molecular marker based study also

Table 3. Similarity coefficient between genotypes using 20 ISSR profiles

Sl.No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1.00																			
2	0.75	1.00																		
3	0.69	0.71	1.00																	
4	0.50	0.52	0.46	1.00																
5	0.68	0.84	0.77	0.44	1.00															
6	0.52	0.54	0.54	0.35	0.59	1.00														
7	0.54	0.56	0.62	0.45	0.60	0.48	1.00													
8	0.55	0.57	0.63	0.42	0.68	0.38	0.58	1.00												
9	0.64	0.61	0.67	0.52	0.59	0.48	0.70	0.58	1.00											
10	0.48	0.62	0.56	0.52	0.67	0.35	0.57	0.52	0.50	1.00										
11	0.41	0.52	0.57	0.42	0.50	0.38	0.73	0.43	0.58	0.52	1.00									
12	0.56	0.69	0.75	0.34	0.74	0.41	0.54	0.50	0.48	0.59	0.55	1.00								
13	0.26	0.22	0.38	0.28	0.29	0.29	0.35	0.32	0.30	0.35	0.32	0.41	1.00							
14	0.45	0.52	0.58	0.35	0.56	0.50	0.67	0.54	0.52	0.35	0.54	0.50	0.37	1.00						
15	0.44	0.46	0.46	0.56	0.38	0.42	0.52	0.42	0.46	0.39	0.55	0.39	0.35	0.48	1.00					
16	0.36	0.37	0.48	0.42	0.40	0.30	0.41	0.38	0.42	0.35	0.38	0.41	0.38	0.43	0.59	1.00				
17	0.35	0.36	0.36	0.26	0.28	0.35	0.27	0.20	0.24	0.33	0.25	0.35	0.27	0.29	0.33	0.28	1.00			
18	0.44	0.41	0.46	0.33	0.44	0.59	0.45	0.31	0.40	0.39	0.31	0.50	0.28	0.41	0.40	0.42	0.41	1.00		
19	0.39	0.36	0.41	0.40	0.33	0.29	0.33	0.31	0.30	0.33	0.31	0.34	0.44	0.35	0.56	0.59	0.50	0.40	1.00	
20	0.42	0.44	0.50	0.24	0.42	0.67	0.50	0.33	0.43	0.25	0.39	0.42	0.24	0.45	0.37	0.25	0.47	0.53	0.24	1.00

Where, 1= D. Sel.-1, 2= D. Sel.-5, 3 = D. Sel.-6, 4 = Wright, 5= HFO 114, 6= OL 125, 7 = UPO 265, 8 = UPO 270, 9 = UPO 271, 10 = UPO 273, 11 = UPO 275, 12 = Kent, 13 = UPO 212, 14 = No. 1, 15 = OS 6, 16 = EC 605833, 17 = EC 605836, 18 = EC 605838, 19 = UPO 260, 20 = EC 246199

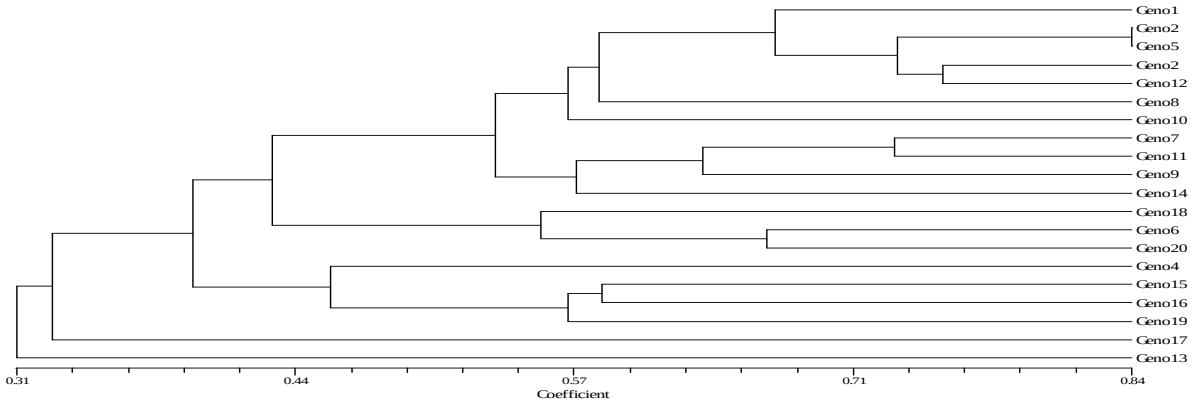

Fig 1. Dendrogram obtained from the pooled data of 20 ISSR profiles and 20 genotypes of oat, where, Geno = Genotype and are numbered same as in table 3.

Table 4. Dissimilarity coefficient between genotypes using 8 quantitative variables rainfed plots

Sl.No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	0.000																			
2	0.175	0.000																		
3	0.339	0.175	0.000																	
4	0.136	0.180	0.018	0.000																
5	0.288	0.006	0.122	0.265	0.000															
6	0.005	0.222	0.022	0.155	0.330	0.000														
7	0.267	0.106	0.011	0.248	0.119	0.314	0.000													
8	0.235	0.007	0.010	0.212	0.108	0.283	0.004	0.000												
9	0.134	0.105	0.010	0.103	0.181	0.168	0.195	0.156	0.000											
10	0.219	0.085	0.085	0.178	0.141	0.264	0.007	0.004	0.142	0.000										
11	0.387	0.218	0.022	0.343	0.150	0.433	0.128	0.152	0.291	0.176	0.000									
12	0.133	0.163	0.016	0.094	0.267	0.165	0.209	0.182	0.143	0.146	0.319	0.000								
13	0.237	0.101	0.010	0.196	0.782	0.275	0.143	0.111	0.113	0.121	0.200	0.542	0.000							
14	0.116	0.138	0.014	0.114	0.214	0.139	0.236	0.198	0.005	0.186	0.334	0.163	0.149	0.000						
15	0.475	0.311	0.031	0.416	0.233	0.520	0.220	0.242	0.373	0.259	0.010	0.396	0.278	0.417	0.000					
16	0.410	0.299	0.030	0.361	0.312	0.453	0.210	0.233	0.361	0.224	0.206	0.300	0.327	0.406	0.225	0.000				
17	0.312	0.212	0.021	0.266	0.251	0.355	0.143	0.155	0.268	0.136	0.194	0.202	0.251	0.311	0.248	0.010	0.000			
18	0.497	0.373	0.037	0.441	0.362	0.540	0.275	0.302	0.437	0.299	0.229	0.386	0.386	0.482	0.209	0.009	0.187	0.000		
19	0.290	0.120	0.006	0.269	0.000	0.333	0.105	0.010	0.187	0.134	0.138	0.265	0.009	0.222	0.224	0.298	0.239	0.348	0.000	
20	0.310	0.228	0.023	0.247	0.271	0.349	0.173	0.176	0.265	0.147	0.222	0.188	0.258	0.307	0.268	0.120	0.005	0.203	0.796	0.000

Where, 1= D. Sel.-1, 2= D. Sel.-5, 3 = D. Sel.-6, 4 = Wright, 5= HFO 114, 6= OL 125, 7 = UPO 265, 8 = UPO 270, 9 = UPO 271, 10 = UPO 273, 11 = UPO 275, 12 = Kent, 13 = UPO 212, 14 = No. 1, 15 = OS 6, 16 = EC 605833, 17 = EC 605836, 18 = EC 605838, 19 = UPO 260, 20 = EC 246199

Fig 2. Dendrogram obtained from the pooled data of 8 quantitative variables from rainfed plots and 20 genotypes of oats, where, Geno = Genotype and are numbered same as in table 4

indicated similar non-compatible results (Burstin and Charcrosset, 1997; Ben-Har *et al*., 1995) in different crops. The moderate association between genetic distances estimated using molecular and phenotypic markers can be explained by a range of factors. The association between estimates is also influenced by the fact that a large portion of the variation detected by molecular markers is non-adaptive and, therefore, not subject to either natural or artificial selection. On the other hand, the phenotypic characters are subject to both natural and artificial selection, aside from their high environmental dependence. Moreover, the observations taken from rainfed plots were quantitative in nature so naturally covered many minor gene distributed non-uniformly within the genome; also, it is not always the case that two identical phenotypes are determined by the same genes, *i.e.*, distinct genes may lead to similar phenotypes. Thus, it is clear that such estimates are closer when there is an association between the loci controlling the targeted morphological traits (quantitative trait loci, or QTLs) and the evaluated bands and when a large number of qualitative as well as quantitative traits are evaluated (De Lose and Baril. 2001; Roy *et al*., 2004).

Conclusion

Molecular analysis provides a wider genome sampling than the morphological analysis, since a study comparing both the techniques rarely evaluates the same or even a similar number of morphological and molecular markers. It can be concluded from the study that the association technique came with best discrimination among all the genotypes studied (classified the test cultivars into maximum number of clusters) for genetic diversity and association analysis, hence may be considered more efficient marker association technique for oats, when a large number of quantitative and qualitative traits are evaluated. Also, the results can be used to identify desirable parental combination and associate both morphological as well as molecular marker (ISSR) system, which could then be used in oat improvement programme.

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