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Full Length Research Paper

RAPD and SSR analysis of wild oats (*Avena* species) from North West frontier Province of Pakistan

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Genetic diversity of 10 biotypes of wild oats (*Avena* species) which differ in certain morphological and physiological characters were analyzed using 9 Randomly Amplified Polymorphic DNA (RAPD) and 2 Simple Sequence Repeat (SSR) primer sets. RAPD and SSR primers revealed different levels of genetic polymorphisms among the various biotypes. Range of genetic distances estimated by using individual primers was 0 – 100 % but on an average genetic distances were in the range of 30 - 65% indicating that these biotypes were genetically very diverse and possess a high degree of polymorphism.

Key words: Wild oats, DNA, Polymorphism, RAPD, SSR.

INTRODUCTION

Average production of wheat in Pakistan (2 tonns ha^{-1}) is much less than that of developed countries (6 tonns ha^{-1}). One of the important reasons of lower yield of wheat is the problem of weeds. The weeds cause considerable losses to our major crops and cause 25 - 30% losses in most important staple food crop of wheat. Wild oat (*Avena* species) is most serious grassy weed for rabi crops, especially, associated with the wheat crop. It is highly competitive weed with wheat and causes approximately 60% of total weed losses (Khan et al., 2007). Due to crop resemblance with wheat at seedling stage, it is difficult to control it manually; therefore, herbicides application becomes inevitable. Since different biotypes of wild oats belonging to different agro-climatic regions are showing different level of herbicide resistance, thus it is important to recommend herbicide dosage in accordance with genetic diversity of each biotype for each locality.

Genetic diversity is of prime importance for the survival, adaptation to certain agro-climatic conditions, success and improvement of any crop species. Unless there is not enough genetic diversity in the germplasm, it is practically not possible to increase the yield and other desireable characters of the crop, because selection for the improved genotypes depends on the availability of genetic variability within the breeding material.

There is a need to characterize the indigenous germplasm of wild oats (*Avena* species) using available methods. Phenotypic/morphological characterization (previously used) is easy and cheaper way to characterize the germoplasm, but it has its own limitation, because very few morphological characters can be utilized for characterization. Similarly biochemical characterization (Islam and Shepherd, 1992) though successful in many cases but because of limited number of biochemical marker loci (Hart, 1987) the technique could not be used on wider scale. Recent development of molecular genetic techniques, especially RFLP (Paterson et al., 1991), Simple Sequence Repeat (Roder et al., 1998) and RAPD (Williams et al.,1990) have transformed the opportunities of utilizing molecular biology for characterization of crop/ weed species.

Different biotypes of weeds were identified by using RAPD marker (Mitra et al., 1998). RAPDs also require very low amount of total genomic DNA, thus it is easier and faster for generating data (Nissen et al., 1995). Another commonly used marker system is simple sequence repeats (SSRs). The distribution of simple sequence repeats (SSRs) show that SSRs are far more common in plant genomes than previously estimated (Cardle et al., 2000). DNA marker data could be used as selection criteria for bicontrol by providing an estimate of

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Table 1. Different biotypes of Wild oats (*Avena species*) collected from various districts of N.W.F.P. and Islamabad / Rawalpindi.

S. No	Name of Biotypes	Collection Site
1	Dera Ismail Khan White	Tatta Balochan , D.I. Khan
2	Dera Ismail Khan Black	Rakshah Koat , D.I. Khan
3	Karak White	Lakki Ghundakki , Karak
4	Karak Black	Babul Khel , Karak
5	Peshawar	Malkandher Farm , Peshawar
6	Charsadda	Mvliano Kala , Charsadda
7	Mardan	Lundkhawar , Mardan
8	Malakand	Dargai , Malakand
9	Swat	Batkhela , Swat
10	Islamabad / Rawalpindi	NARC , Islamabad

Table 2. Name, Sequence. Size, molecular weight and %GC content of 9 RAPD primers used to study genetic diversity among 10 biotypes of wild oats.

S. No	Name	Sequence	Size	Mol. Wt.	%GC
1.	GLA-03	AGTCAGCCAC	10	2996.98	60
2.	GLA-04	AATCGGGCTG	10	3068.02	60
3.	GLA-12	TCGGCGATAG	10	3068.02	60
4.	GLB-05	TGCGCCCTTC	10	2954.97	70
5.	GLB-19	ACCCCCGAAG	10	2981.97	70
6.	GLC-07	GTCCCGACGA	10	3012.99	70
7.	GLD-17	TTCCCCCCAG	10	2923.95	70
8.	GLD-18	GAGAGCCAAC	10	3046.00	60
9.	GLE-05	TCAGGGAGGT	10	3108.04	60

a plants genetic diversity relative to other potential target species (Nissen et al., 1995). So far no such work has been documented in Pakistan. In the present study, genetic diversity of 10 biotypes of wild oats (*Avena* species) which differ in certain morphological and physiological characters were analyzed using 9 Randomly Amplified Polymorphic DNA (RAPD) and 2 Simple Sequence Repeat (SSR) primer sets.

MATERIALS AND METHODS

Plant materials

Ten biotypes of wild oats (*Avena* species) supplied by the Department of Weed Science, Agricultural University Peshawar-Pakistan were used in present study. Table 1 shows list of collected biotypes along with their collection sites. All the materials were planted in the green house at the Institute of Biotechnology and Genetic Engineering (IBGE), N.W.F.P. Agricultural University, Peshawar.

DNA extraction

DNA was extracted from leaf of wild oats as described by Weining and Langridge (1991). Explain the method briefly, that is, the medium used etc. To remove RNA, DNA was treated with 40 microgram RNAse-A (Gene link, USA) at 37℃ for 1 h, then DNA samples were run on 0.8% Agarose gel to check the quality and quantity of DNA and stored at 4℃. To use in Polymerase Chain Reaction (PCR) a 1:5 dilutions of DNA were made in doubled distilled, deionized and autoclaved water.

Polymerase chain reaction

The PCR was carried out using protocols of Devos and Gale (1992) with slight modification. All amplification reactions were performed using GeneAmp PCR System 2700 (Applied Biosystem) programmable thermocycler. Simple Sequence Repeats (SSRs) primer sets specific for wheat chromosomes (Roder et al., 1995; Liu et al., 1999) were also used for *Avena* species (wild oats) DNA analysis in order to find any homology of DNA sequences between the two crops and to check preliminary results that SSR primer sets specific for wheat chromosomes can work for wild oats or not. All amplification reactions were performed using GeneAmp PCR System 2700 (applied biosystem) programmable thermocycler. The amplification products of RAPDs primers were separated on 1.5% and SSRs primer sets on 3% agarose / TBE Gel added, then visualized by staining with Ethidium Bromide under ultra-violet (U.V) light and photographed using gel documentation system “Uvitec”. The information regarding nine Decamer Randomly Amplified Polymorphic DNA (RAPD) primers and two SSRs primer sets are given on Table 2 and 3 respectively.

For statistical analysis, each band was considered as single

Table 3. Name, Sequence, Repeats and melting temperatures of 2 SSR primers sets used to study genetic diversity among 10 biotypes of wild oats (Size range of SSR products< 250 - 750bp.

S. No	Locus	Sequence (Forward primer)	Sequence (Reverse Primer)	Repeats	T_m
1	*Xgdm86-2B*	GGTCACCCTCTCCCATCC	GCGCTCCATTCAATCTG	(TG)13	60
2	*Xgdm87-2D*	AATAATGTGGCAGACAGTCTTGG	CCAAGCCCCAATCTCTCTCTCT	(GT)14	60

T_m = Melting Temperature, (Annealing temperature = T_m – 5°C

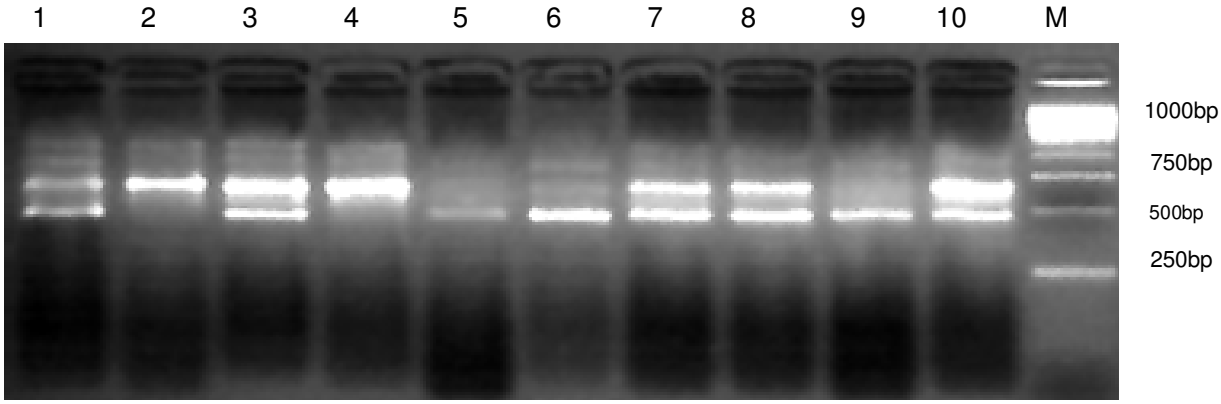

Figure 1. PCR Amplification profile of 10 wild oats biotypes using RAPD primer GL-A12. From left to right: 1 = Dera Ismail Khan White, 2 = Dera Ismail Khan Black, 3 = Karak White, 4 = Karak Black, 5 = Peshawar, 6 = Charsadda, 7 = Mardan, 8 = Malakand, 9 = Swat and 10 = Islamabad and M = Molecular size of I kb ladder DNA (size of fragments are indicated in bp on right).

locus / allele. Only scorable bands were included in the analysis. Bands were scored as present (1) / absent (0) and bi-variate (1 - 0) data matrix was generated. Genetic distances were calculated using Unweighted Pair Group of Arithmetic Means (UPGMA) procedure as suggested by Nei and Li (1979).

$$GD_{xy} = 1 - N_{xy} / N_x + N_y - N_{xy}$$

Where GD_{xy} = Genetic distance between two genotypes
N_{xy} = Total number of common bands in the two genotypes
N_x = Number of bands in genotype-1
N_Y = Number of bands in genotype-2.

Genotypes were clustered using computer programme "Popgene 32" version 1.31 (http://www.ualberta.ca./~fyeh/fyeh). Based on these analysis dendrograms were generated for RAPD and SSR primers sets separately to estimate diversity among 10 biotypes of wild oats.

RESULTS AND DISCUSSION

RAPD analysis

Genetic diversity among 10 biotypes of wild oats (*Avena* species) were estimated using 9 RAPD primers (Table 2).The banding pattern obtained by using RAPD primer Genelink (GL)-A03, the 10 biotypes of wild oats showed various levels of genetic polymorphism. A total of 21 alleles (bands) were amplified in 10 biotypes giving an average of 2.1 alleles per biotype. The size of amplified fragments, estimated by using 1 kb DNA ladder, ranged from >250 bp to >1000 bp. Range of genetic distances among biotypes was 0 to 75%. Maximum genetic distance (75%) was estimated for 4 comparisons viz Dera Ismail Khan (black) - Dera Ismail Khan (white), Dera Ismail Khan (black) - Peshawar, Dera Ismail Khan (black) - Islamabad and Peshawar - Swat, while 4 comparisons showed homozygosity for the loci amplified by primer GL-A03 (data not shown).

The PCR amplification profile using GL -A04 primer (data not shown), showed various levels of genetic polymorphism for the loci amplified. Total genomic DNA of 2 biotypes (Malakand and Islamabad) was not amplified by using GL-A04 and so the results are not included in analysis. A total of 32 alleles were observed in 8 biotypes giving an average of 4 alleles per biotype. The size of amplified fragments ranged from < 500 bp to >1000 bp. Range of genetic distances estimated was 28 to 100%. Maximum genetic distance (100%) was observed for 3 comparisons, Dera Ismail Khan (white) - Swat, Karak (black) - Swat and Mardan - Swat, while one comparison: Dera Ismail Khan black - Karak white showed 72% homozygosity for the amplified loci.

The PCR amplification profile of 10 biotypes using GL - A12 primer (Figure 1) yielded a total of 24 alleles giving an average of 2.4 alleles per biotype. The size of amplified fragments varied from 500 to 1000 bp. Genetic distances estimated for all the possible combinations

ranged from 0 to 100%. Four pairs of biotypes Dera Ismail Khan (black) - Peshawar, Dera Ismail Khan (black) - Swat, Karak (black) - Peshawar and Karak (black) - Swat showed maximum genetic distance (100%), while three comparisons Dera Ismail Khan (black) - Karak (black), Charsadda - Islamabad and Mardan - Malakand revealed no difference (GD = 0%)

The PCR amplification profile of 10 biotypes of wild oats using GL-B07 primer showed various levels of genetic polymorphism. A total of 38 alleles (bands) were amplified in 10 biotypes giving an average of 3.8 alleles per biotype. The size of amplified fragments ranged from 500 to 1000 bp. Range of genetic distances observed using RAPD GL-BO7 was 0 to 60%. Six comparisons showed 60% genetic dissimilarity while 9 comparisons showed no genetic difference using RAPD primer GL-B07.

A total of 37 alleles were amplified using RAPD primer GL -B19 in 10 biotypes giving an average of 3.7 alleles per biotype. The size of amplified fragments ranged from >250 to 1000 bp. Genetic distances (GD) among the 10 biotypes ranged from 0 to 60 %. Maximum genetic distance (60%) was estimated for 2 comparisons (Dera Ismail Khan white – Peshawar and Karak black - Peshawar), while 4 comparisons viz Dera Ismail Khan (black) - Karak (White), Mardan - Malakand, Mardan - Islamabad and Malakand - Islamabad showed homozygosity for the loci amplified using GL-D17.

A total of 43 alleles (bands) were amplified in 9 biotypes using GL-C07 giving an average of 4.8 alleles per biotype. Total genomic DNA of one biotype (Islamabad) was not amplified using GL-C07 and so the result is not included in this analysis. The size of amplified fragments ranged from >500 to >1000 bp. Range of genetic distances among biotypes estimated was 14 to 86%. Maximum genetic distance (86%) was observed for one comparison (Peshawar - Swat), while two comparisons (Dera Ismail Khan White - Peshawar and Dera Ismail Khan Black - Peshawar) were only 14% heterozygous for the detected loci.

The banding pattern of wild oats biotypes obtained using GL -D17 primer showed various levels of genetic polymorphism. Total genomic DNA of one biotype (Swat) was not amplified using GL-D17 and so the result is not included in this analysis. A total of 21 alleles (bands) were amplified using 9 biotypes giving an average of 2.3 alleles per biotype. The size of amplified fragments varied from 500 bp to approximately 1000 bp. Genetic distances estimated for all the possible combinations ranged from 0 to 75%. Two pairs of biotypes (Dera Ismail Khan (white) - Mardan and Charsadda - Mardan showed maximum genetic distance (75%), while four pairs (Dera Ismail Khan (white) - Charsadda, Karak (black) - Malakand, Karak (black) - Islamabad and Malakand - Islamabad) revealed no difference (GD = 0%) in the loci detected using GL-D17 primer.

The amplification PCR profile of 10 biotypes of wild oats (*Avena* species) using GL-D18 primer amplified a total of 40 alleles in 10 biotypes giving an average of 4 alleles per biotype. The size of amplified fragments varied from 500 to > 1000 bp. Range of genetic distances observed using RAPD GL-D18 primer was 0 to 100%. One comparison (Malakand - Swat) showed 100% genetic dissimilarity, while 3 comparisons (Dera Ismail Khan white - Dera Ismail Khan black, Dera Ismail Khan white - Karak white and Dera Ismail Khan black - Karak white) showed no genetic difference using RAPD primer GL-D18.

A total of 16 alleles (bands) were observed in 7 biotypes giving an average of 2.6 alleles per biotype using Genelink-E05 primer. Total genomic DNA of 3 biotypes (Charsadda, Swat and Islamabad) was not amplified using GL-E05 and so the result is not included in analysis. Total genomic DNA of 3 biotypes (Charsadda, Swat and Islamabad) were not amplified using GL-E05 and so the result are not included in analysis The size of amplified fragments varied from > 250 to 1000 bp. Range of genetic distances estimated was 0 to 33%. Maximum genetic distance (33%) was observed for 10 comparisons, while 11 comparisons were completely homozygous for the detected loci.

Average genetic distances of 10 biotypes of wild oats using 9 RAPD primers ranged from 24 to 64.14% (Table 4). Maximum average genetic distance (64.14%) were noted for one comparasion (Dera Ismail Khan Black – Swat), closely followed (63.14%) by (Peshawar – Swat), while minimum average genetic distance (24.67%) was recorded for one comparison (Dera Ismail Khan white – Islamabad), closely followed (27.50%) by one comparison (Mardan and Malakand). Generally average genetic distances were in the range of 30 - 50%.

SSR analysis

Simple PCR amplification profile of 10 biotypes of wild oats (*Avena* species) using SSR primer set gdm 86-2B SSR showed various levels of genetic polymorphism among the biotypes at DNA level. Total genomic DNA of 3 biotypes (Dera Ismail Khan White, Mardan and Swat) was not amplified using SSR 86-2B (their products don't give amplification due to unknown reason that may be due to misuse) and so the results are not included in analysis. A total of 12 alleles were amplified in 7 biotypes giving an average of 1.2 alleles per biotype. The size of amplified fragments were in the range of < 250 bp. Estimates of genetic distances ranged from 0 - 50%. Eleven comparisons showed no differences for the loci amplified using gdm 86-2B, while 10 comparisons revealed maximum (50%) genetic dissimilarities.

Ten biotypes of wild oats (*Avena* species) yielded a total of 31 alleles, giving an average of 3.1 alleles per biotype using SSR primer set gdm-87-2D (Figure 4). The

Table 4. Average genetic distances (in percentage) among 10 *Avena* Species (wild oats) based on 9 RAPD Primers.

Biotyes	D.I.K (W)	D.I.K (B)	KRK (W)	KRK (B)	PESH	CHRD	MRD	MKD	SWT
D.I.K. (W)	-								
D.I.K. (B)	36.67	-							
KRK (W)	31.67	28.00	-						
KRK (B)	33.67	25.89	39.33	-					
PESH	47.11	49.44	54.78	43.78	-				
CHRD	29.5	48.38	45.75	43.38	46.25	-			
MRD	56.67	45.78	44.33	48.78	34.56	45.75	-		
MKD	42.88	40.88	42.50	39.00	31.50	29.71	**27.50**	-	
SWT	56.57	**64.14**	61.57	54.71	**63.14**	56.28	61.57	62.00	-
ISLM	**24.67**	40.16	42.83	31.67	34.50	28.00	43.16	26.16	39.20

D.I. K (W) = Dera Ismail Khan White, D.I. K (B) = Dera Ismail Khan Black, KRK (W) = Karak White, KRK (B) = Karak Black, PESH = Peshawar, CHRD = Charsadda, MRD = Mardan, MKD = Malakand, SWT = Swat, ISLM = Islamabad / Rawalpindi.

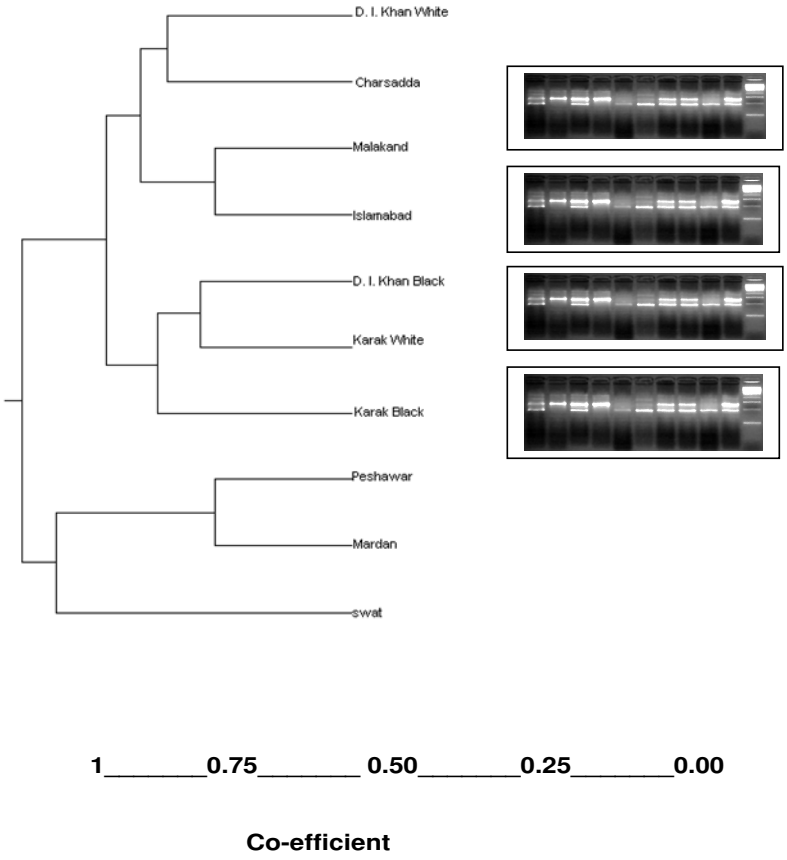

Figure 2. Dendrogram of 10 biotypes of Wild oats constructed by using data obtained 9 RAPD primers.

size range for the amplified bands was from < 250 – 750 bp. The estimated genetic distance among the comparisons ranged from 0 - 100%. Maximum genetic distance (100%) was observed for 7 comparisons while one comparison (Karak black - Islamabad) showed homozygosity for the loci amplified by using SSR primer gdm 87-2B.

Cluster analysis

The dissimilarity coefficient matrix of 10 biotypes of wild oats based on the data of 9 RAPD primers using UPGMA method (Nei and Lie, 1979) was used to construct a dendrogram using computer program “popgene” {(http://www.ualberta.ca./~fyeh/fyeh) Figure 2 }. The

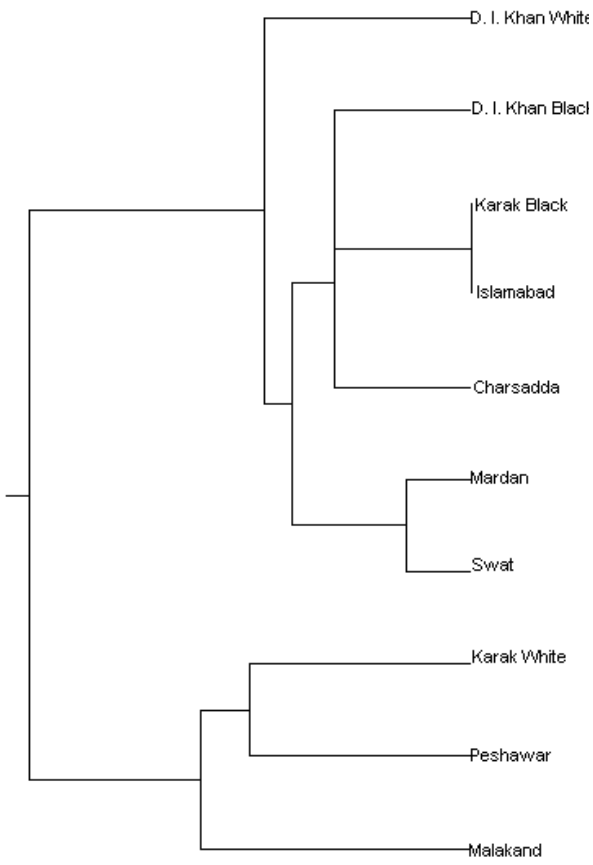

Figure 3. Dendrogram of 10 biotypes of Wild oats constructed by using data obtained 2 SSR primer set.

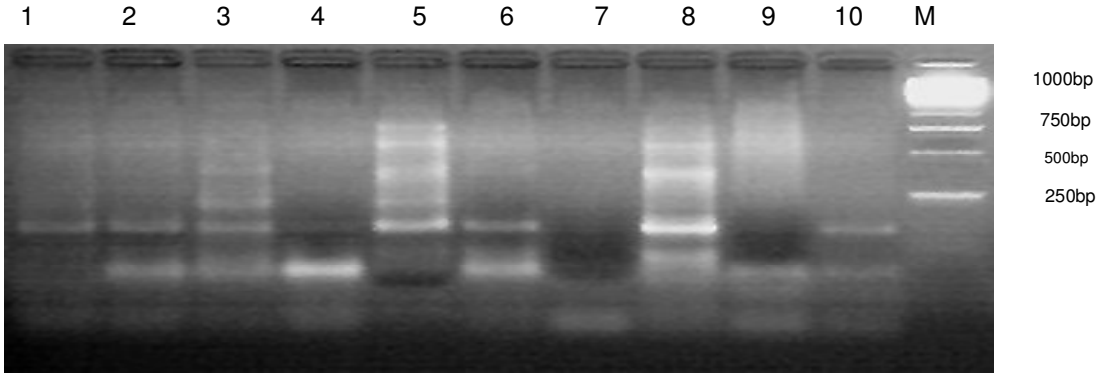

Figure 4. PCR Amplification profile of 10 wild oats biotypes using SSR primer dgm 87-2D.

biotypes were grouped in two main groups ("A" and "B"). Group A was the largest (comprising of 7 biotypes) and was subdivided into three subgroups "C", "D" and "E". Group "B" was smallest and comprised of 3 biotypes only. Based on the dendrogram analysis, Dera Ismail Khan white biotype (representing semi-arid region) and Swat biotype (representing humid region) were most distantly related to each other. This finding was further strengthened by average genetic diversity analysis (Table 4) where the two biotypes showed higher levels of genetic dissimilarity with rest of the biotypes used during present study. This further supported the hypothesis that

cluster analysis can reliably be used for identification of diverse biotypes of weeds which may be utilized in weed management and breeding programs.

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