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GENETIC FINGERPRINTING OF LOCAL TURMERIC GENOTYPES USING RAPDS

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Abstract

Genetic fingerprinting of turmeric (*Curcuma longa* L.) genotypes collected from 5 different regions of Bannu including Ismail Khel, Kaki, Raakh Sarkar, Michan Khel and Mandan was performed using Randomly Amplified Polymorphic DNA (RAPD). A total of 22 primer were evaluated for genetic diversity studies, of which 3 (OPE-07, OPC-01 and OPA-03) were found suitable. In total these generate 141 fragments, of which 40 fragments were polymorphic with 28.36% of polymorphism. The number of amplification products generated by each primer varied from 6 (OPC-01) to 17 (OPA-03). The polymorphism of turmeric genotypes using OPA-03 (38.3%) was found highest followed by OPE-07 (25.0%) and OPC-01 (21.74%). It was observed that OPA-03 was better to discriminate genotype as compared to other markers. Un-weighed Pair Group Arithmetic Averages (UPGMA) analysis has clustered 50 turmeric genotypes into 6 groups showing their differentiation on the basis of their locality. On the basis of population locality cluster analysis clustered all the collected turmeric populations of Bannu region into two groups including Group I and Group II. Group I include four populations P1, P4, P5 and P2 collected from Ismail Khel, Michen Khel, Mandan and Kaki respectively while Group II was found unique as it comprised of only one population P3 form Raakh Sarkar. Turmeric genotypes evaluated using RAPD in the present study is helpful to identify potential genotypes which give broadness to the germplasm base of turmeric breeding programs.

Introduction

Curcuma is the member of the family *Zingiberaceae* which have wide medicinal and industrial applications. There are about 100 species of genus Curcuma found in the tropics of Asia which extends from Africa to Australia. The highest diversity is noticed in India and Thailand, with at least 40 species in each area, followed by Burma, Bangladesh, Indonesia and Vietnam (Jan *et al*., 2011). The ecology of the species varied so much that their habitat ranges from sea level (sandy coastal habitat) to high altitude such as above 2000 m in the Himalayas. Daod & Aslam, (1996) have suggested that *C. longa* has been widely cultivated at the mass level in Pakistan in the regions of Kasur, Sahiwal, Okara, Bannu, Pubbi and Haripur areas of Pakistan (Jan *et al*., 2012).

The decreasing number of natural populations and rapid fragmentation of natural habitat may have severe impact on the genetic potential of this species (Paisooksantivatana *et al*., 2001). Besides this, taxonomic identity of the species is also confusing. Their potential use, genetic diversity at species and population level are also unexplored. Taxonomic identity of the species is important to search and confirm the origins of different potential. Extending to that knowledge of genetic diversity it will greatly help to utilize and conserve *Curcuma longa* genetic resource of the country. Even though germplasm collections represent the main source of variability for turmeric genetic improvement studies which is mostly restricted to phenotypic makers. However, the use of phenotypic traits in germplasm characterization may be limited due to the small number of descriptors available and the influence of environment and genotype x environment interactions. Molecular marker techniques may overcome many limitations of the morphological and biochemical markers for the discrimination of turmeric cultivars by providing genetic background for the observed phenotypic variability since they are not affected by the environment or developmental stage and can detect variation at the DNA level (Tingey & Del-Tufo, 1993).

Molecular markers employed for characterization of inter and intra specific diversity of turmeric cultivars include ISSRs, SSRs and chloroplast or nuclear markers (matK, rbcL, rpl16 and trnK) (Pervaiz *et al*., 2010). Hussain *et al*., (2008) studied RAPD analysis for 30 accessions of 5 *Curcuma* species including *C. latifolia, C. malabarica, C. manga, C. raktakanta* and 13 *C. longa* conserved morphotypes. Their RAPD data corroborated the morphological classification of the morphotypes. The efficiency of individual RAPD primers was also compared showing that RAPD markers were highly informative in discriminating the germplasm of *Curcuma*. Pinheiro *et al*., (2003) attempted to use random amplified polymorphic DNA (RAPD) markers to screen 20 turmeric accessions from Brazil, providing useful insights into the structure of genetic diversity. However, RAPD markers are known to yield low levels of polymorphism when compared to other molecular markers, such as microsatellites (simple sequence repeats, SSR) (Powell *et al*., 1996; Akbar *et al*., 2011). Since knowledge of the genetic variation in the turmeric germplasm is essential to increase the efficiency of selection in breeding programs, as well as to direct conservation strategies in germplasm collections. So the present study is aimed to evaluate genetic diversity and relationships among natural populations of *C. longa* on the basis of RAPD markers.

Materials and Methods

A total 50 accessions were collected from 5 different sampling areas in Bannu namely Ismail Khel, Kaki, Raakh Sarkar, Michan Khel and Mandan.The sampling was done from August to September 2011. Information about sampling areas including geographical descriptions and environmental parameters are presented in Fig. 1 and Table 1.

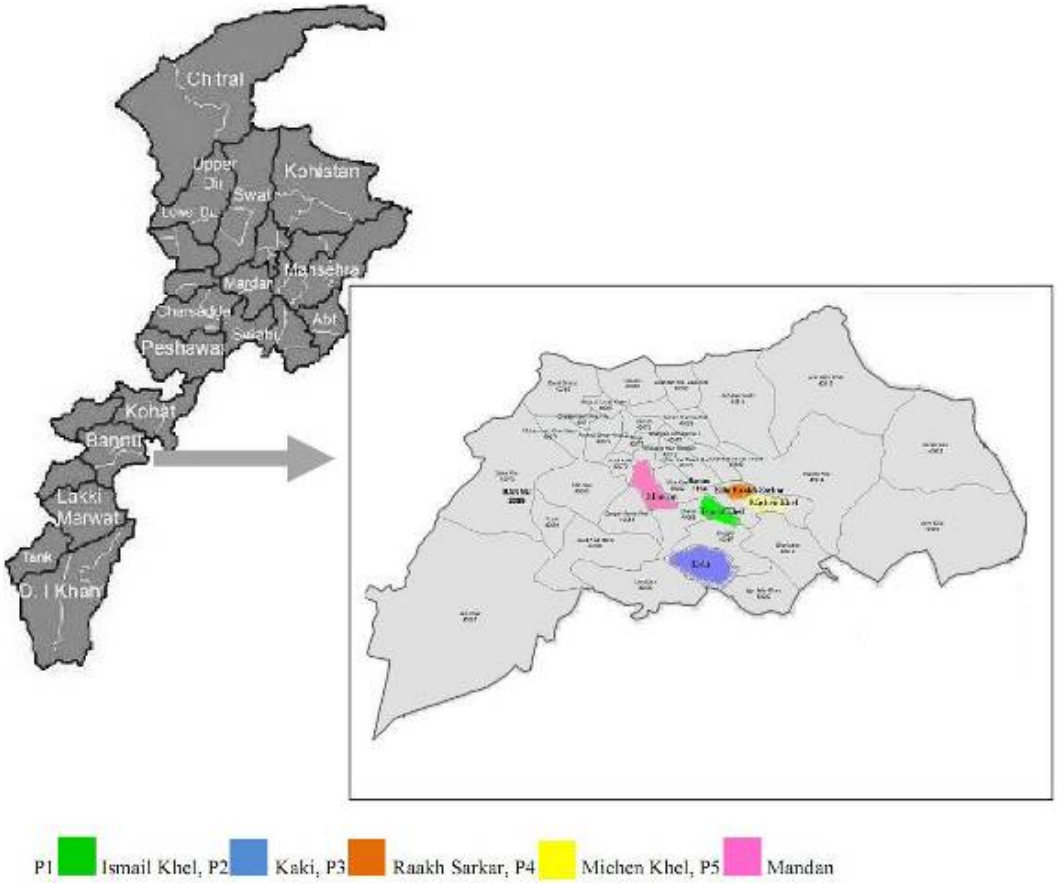

Fig. 1. Study area showing different regions of Bannu for the collection of turmeric genotypes.

Table 1. Map showing the area of collection for Turmeric genotypes and their environmental parameters.

Sampling areas	Sampling codes	District	Latitude	Longitude	Temperature °F		Average rainfall(mm)
					max	min	
Ismail Khel	P1	Bannu	32° 56' N	70° 39' E	84	60	416
Kaki	P2	Bannu	25° 63' N	88° 63' E	84	60	416
Raakh Sarkar	P3	Bannu	23° 46' N	90° 23' E	84	60	416
Michan Khel	P4	Bannu	22° 35' N	91° 42' E	84	60	416
Mandan	P5	Bannu	22° 21' N	91° 50' E	84	60	416

Turmeric leaf samples were collected and immediately stored in silica bags. These samples were processed for DNA isolation using modified protocol of Doyle & Doyle (1990). Around 0.1 gm of turmeric leaf samples were crushed in liquid nitrogen. After crushing the leaf samples were processed with the 900 µl extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl, 0.07% 2-mercaptoethanol) and incubated at 65 °C for overnight in the presence of 5 µl β-mercaptoethanol. After the addition of 600 µl chloroform-isoamyl alcohol, these tubes were centrifuged at 12000 rpm for 5-10 minutes at 4°C. After centrifugation, 500 µl aqueous layer were treated with 100 µl NaI and 1.5 volume of Ethanol and incubated at -20°C for at least 2 hours. After centrifugation at 12000 rpm for 5-10 minutes at 4°C, supernatant was discarded and pallet was washed with 800 µl wash solution (70% ethanol). This step was repeated twice and then air dried for 20 minutes at room temperature. The pellet was re suspended in 30 µl TE buffer and treated with RNAse. The DNA concentration was determined by comparing band intensity with λDNA standards of known concentration under UV light on 1.0% (w/v) agarose gel electrophoresis with ethidium bromide staining. The DNA samples were stored at -20°C at working solutions of genomic DNA (25 µg/µl). A total of 22 random primers (OPA-03, OPC-01, OPC-05, OPC-10, OPC-09, OPC-13, OPC-20, OPD-07, OPE-07, OPF-07, OPI-16, OPI-19, OPJ-19, OPK-17, OPK-19, OPL-03, OPM-14, OPO-06, OPO-10, OPO-11, OPO-16, OPO-13) were selected for RAPD analysis from Operon technologies as shown in Table 2.

Table 2. RAPD-PCR Primers sequences used for the amplification of DNA from Turmeric genotypes.

Primers	[Sequences 5'to 3']	Size range (bp)	Tm (^oC)
OPA-03	[AGTCAGCCAC]	1500-3200	33
OPC-01	[TTCGAGCCAG]	1000-3200	33
OPC-05	[TGGACCGGTG]	1000-3300	33
OPC-09	[CTCACCGTCC]	1500-3000	33
OPC-10	[TGTCTGGGTG]	900-3000	33
OPC-13	[AAGCCTCGTC]	100-1000	33
OPC-20	[ACTTCGCCAC]	500-3000	33
OPD-07	[TTGGCACGGG]	700-3400	33
OPE-07	[AGATGCAGCC]	1003-277	33
OPF-07	[CCGAATTCCC]	1100-3000	33
OPI-16	[TCTCCGCCCT]	1200-3300	33
OPI-19	[AATGCGGGAG]	700-3000	33
OPJ-19	[GGACACCACT]	500-3400	33
OPK-17	[CCCAGCTGTG]	400-2500	33
OPK-19	[CACAGGCGGA]	300-2000	33
OPL-03	[CCAGCAGCTT]	1500-3300	33
OPM-14	[AGGGTCGTTC]	100- 900	33
OPO-06	[CCACGGGAAG]	500-3300	33
OPO-10	[TCAGAGCGCC]	500-2500	33
OPO-11	[GACAGGAGGT]	500-2500	33
OPO-13	[GTCAGAGTCC]	1000-3300	33
OPO-16	[TCGGCGGTTC]	700-3000	33

Polymerase Chain Reaction (PCR) was optimized for amplification in a thermocycler (Veriti 96 wells) for about 25 µl reaction volume containing about 1 X PCR buffer (10 mM Tris-HCl; pH 8.3; 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM each of deoxynucleotide triphosphate (DNTPs) mix, 0.4 µM primer, 1 unit Taq DNA polymerase. The thermocycler was programmed for an initial denaturation step of 3 minutes at 94°C followed by 40 cycles of denaturation for 45 s at 95°C, 45 s at 28°C for primer annealing and 45 s at 72°C for primer extension and a final extension cycle of 7 minutes at 72ºC and a hold temperature of 4°C at the end.

Amplified products were separated on 0.5 µg/ml ethidium bromide containing 1.5% agarose gel in 1 X TBE (10 mM Tris-Borate, 1 mM EDTA) buffer at 50 V for 3 hours. After electrophoresis amplified products were visualized at 260nm wavelength on transilluminator and photographed by a gel documentation system. The scoring was done for samples where the bands will be clearly visible and amplified products were reproducible over 2 repeated amplifications. Amplified fragments was scored for the presence (1) and absence (0) of bands and binary matrices was subjected to statistical analyses using NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System version) by Exceter software (Rohlf, 2000). Relationship among accessions of *Curcuma longa* collected from different geographical regions of Pakistan was investigated for effective conservation and utilization of germplasm in crop improvement programs. Pair-wise genetic similarities among and within the curcuma populations was computed using genetic similarity coefficients (Nei, 1973) and corresponding dendrograms of genetic relatedness was constructed by applying Unweighted Pair Group Method with Arithmetic mean (UPGMA) clustering algorithm.

Efficacy of RAPD primers used in diversity analysis of the *Curcuma* accessions was analyzed using the information obtained from profile data of each primer. Diversity parameters for the genetic data of *Curcuma* accessions was compared for estimation of number of polymorphic bands; number of monomorphic bands; average number of polymorphic bands and average number of patterns per primer (I) (Belaj *et al*., 2003).

Results and Discussion

In the present study DNA, isolated with the DNA extraction protocol especially modified for turmeric leaf samples, ranged from 16 ng/µl to 30 ng/µl while DNA quality was from 1.08 to 1.89 and averaged 1.31 for all the samples as shown in Fig. 2. Khan *et al*., (2007) evaluate DNA extraction protocol for DNA isolation from medicinal plants with some modifications and found that protocol was efficient for DNA isolation from fresh and dried samples.

A total of 22 primers were selected for RAPD analysis, of which 3 (OPE-07, OPC-01 and OPA-03) were found useful for Turmeric fingerprinting which revealed in total 141 bands. The molecular size of the bands ranged from 10kb to 50kb. The numbers of bands produced by each primer, the number of polymorphic bands and the percentage of polymorphism are shown in Table 3. It was observed that total bands were observed highest for OPE-07(48.00) followed by OPA-03 (47.00) while it was found

lowest for OPC-01 (46.00). Electrophoregram of RAPDs primers evaluated for Turmeric genotypes are shown in Fig. 3, Fig. 4 and Fig. 5 respectively. Polymorphic bands percentage was found highest for OPA-03 (38.30) while it was found lowest for OPC-01 (21.74). Polymorphism for different Turmeric populations using OPC-01 were observed highest for P4S which revealed 11 bands followed by 10 for P3S and P5S while it was found lowest for P2S which revealed 6 bands. Similarly polymorphism was found highest for P5S (40.00%) followed by P2S (33.33%) while it was found lowest for P4S (9.09%) as shown in Table 4. Polymorphism for different Turmeric populations using OPE-07 were observed highest for P1S, P2S and P4S which revealed 10.00 bands while it was found lowest for P3S and P5S which revealed 9.00 bands. Similarly polymorphism was found highest for P4S (40.00%) followed by P3S, P5S (33.33%) while it was found lowest for both P1S and P2S (10.00%) as shown in Table 5. Polymorphism for different Turmeric populations using OPA-03 were observed highest for P3S which revealed 17.00 bands followed by both P4S and P5S that revealed 8.00 bands while it was found lowest for both P1S and P2S which revealed 7.00 bands. Similarly polymorphism was found highest for P3S (82.35%) followed by P2S (57.14%) while it was found lowest for both P1S and P5S (0.00%) as shown in Table 6.

Fig. 2. Agarose gel electrophoresis of DNA from different turmeric genotypes.

Table 3. Polymorphism of local Turmeric genotypes using different RAPDs primers.

Primers	Total Bands	Monomorphic bands	Polymorphic bands	Monomorphic bands %	Polymorphic bands %
OPC-01	46.00	36.00	10.00	78.26	21.74
OPE-07	48.00	36.00	12.00	75.00	25.00
OPA-03	47.00	29.00	18.00	61.70	38.30
Total	141 ± 1.00	101 ± 4.04	40 ± 4.16	214.96 ± 8.77	85.04 ± 8.77

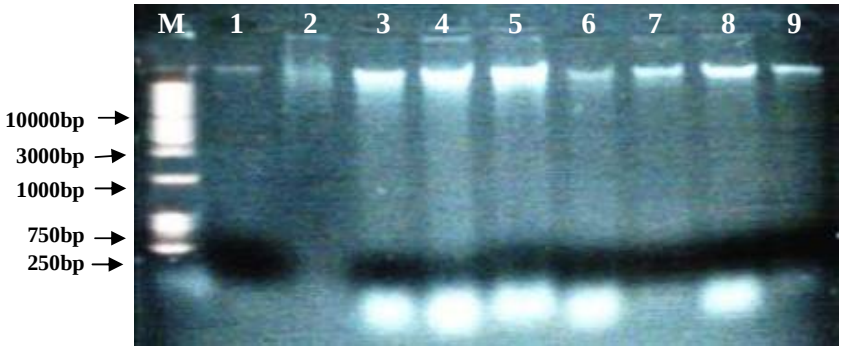

Fig. 3. Agarose gel electrophoresis of PCR products for turmeric genotypes using primer OPC-01.

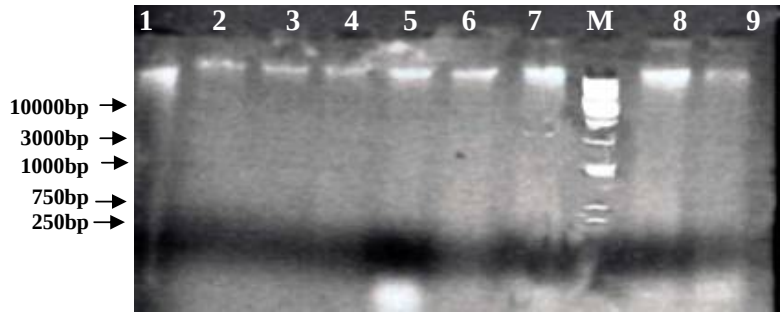

Fig. 4. Agarose gel electrophoresis of PCR products for turmeric genotypes using primer OPE-07.

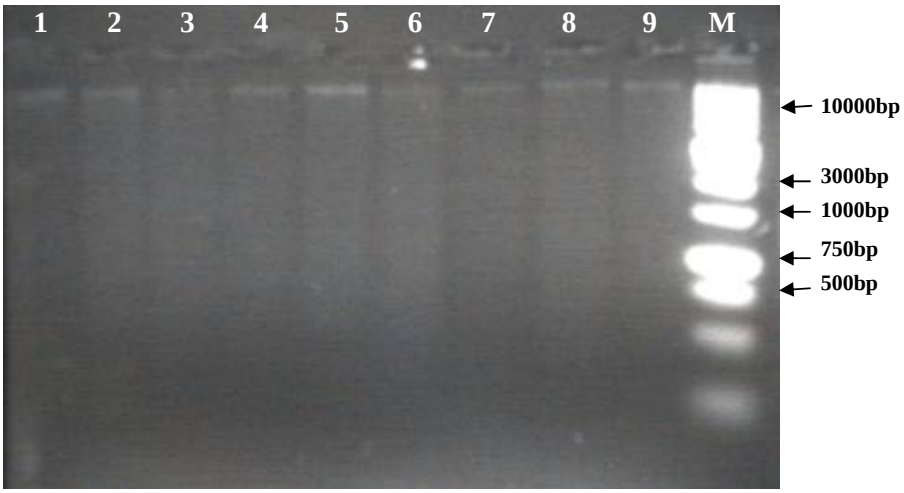

Fig. 5. Agarose gel electrophoresis of PCR products for turmeric genotypes using primer OPA-03.

Table 4. Polymorphism of different Turmeric populations using OPC-01.

Samples	Total Bands	Monomorphic bands	Polymorphic bands	Monomorphic bands %	Polymorphic bands %
P1S(1-10)	9.00	8.00	1.00	88.88	11.11
P2S(1-10)	6.00	4.00	2.00	66.66	33.33
P3S(1-10)	10.00	8.00	2.00	80.00	20.00
P4S(1-10)	11.00	10.00	1.00	90.90	9.09
P5S(1-10)	10.00	6.00	4.00	60.00	40.00
Average	9.20 ± 1.92	7.20 ± 2.28	2.00 ± 1.22	77.29 ± 13.59	22.71 ± 13.59

Table 5. Polymorphism of different Turmeric populations using OPE-07.

Samples	Total Bands	Monomorphic bands	Polymorphic bands	Monomorphic bands %	Polymorphic bands %
P1S(1-10)	10.00	9.00	1.00	90.00	10.00
P2S(1-10)	10.00	9.00	1.00	90.00	10.00
P3S(1-10)	9.00	6.00	3.00	66.67	33.33
P4S(1-10)	10.00	6.00	4.00	60.00	40.00
P5S(1-10)	9.00	6.00	3.00	66.67	33.33
Average	9.60 ± 0.55	7.20 ± 1.64	2.40 ± 1.34	74.67 ± 14.26	25.33 ± 14.26

Table 6. Polymorphism of different Turmeric populations using OPA-03.

Samples	No. of Bands	Monomorphic bands	Polymorphic bands	Monomorphic bands %	Polymorphic bands %
P1S(1-10)	7.00	7.00	0.00	100	0.00
P2S(1-10)	7.00	3.00	4.00	42.86	57.14
P3S(1-10)	17.00	3.00	14.00	17.64	82.35
P4S(1-10)	8.00	8.00	0.00	100	0.00
P5S(1-10)	8.00	8.00	0.00	100	0.00
Average	9.40 ± 4.28	5.80 ± 2.59	3.60 ± 6.07	72.10 ± 39.23	27.90 ± 39.23

Thaikert & Yinyong, (2009) also reported genetic diversity by using 19 random primers. Nineteen primers produced 184 scorable bands, out of which 166 were polymorphic. Jan *et al.*, (2011) also used 10 RAPD primers for turmeric genotypes and generated 95 RAPD fragments, of which 92 fragments were polymorphic with 96.84% of polymorphism. Amplified fragment sizes ranged from 200 to 3640 bp. The percentage of polymorphism ranged from a maximum of 100% for 6 random primers to a minimum of 66.67% by OPA 15 while polymorphism of the turmeric genotypes in the present study was 33.96% with primer OPA-03. Nayak *et al*., (2006) also used RAPD markers to characterize genetic diversity among 17 promising cultivars of *C. longa*. Differential polymorphism was noted in 17 cultivars of turmeric genotypes using 20 primers showing variation in the percentage of polymorphic bands from 35.6% in PTS51 to 98.6% in Acc31.

Cluster analysis distributes the turmeric genotypes of Bannu region into 6 groups (Group I, Group II, Group III, Group IV, Group V and Group VI) as shown in Fig. 6. Cladogram revealed that these genotypes were related in all these 6 groups. Group I comprised of in total 21 genotypes, of which population 1 have 7 genotypes (P1S1, P1S2, P1S4, P1S7, P1S8, P1S9, P1S3), population 2 have 2 genotypes (P2S2, P2S5), population 3 have 6 genotypes (P3S1, P3S9, P3S5 , P3S6, P3S7, P3S8), population 4 have 4 genotypes (P4S2, P4S2, P4S3, P4S5) and population 5 have 2 genotypes (P5S1, P5S7). Group II comprised of in total 9 genotypes, of which population 1 have only one genotype (P1S10), population 2 have 2 genotypes (P2S1 and P2S7), population 3 have 2 genotypes (P3S2 and P3S10) and population 4 have only one genotype (P4S6) while population 5 have 3 genotypes (P5S10, P5S5, P5S8). Group III comprised of 7 genotypes including 4 from population 2 (P2S4, P2S8, P2S9, P2S10) and only only one from population 4 (P4S8) while 2 from population 5 (P5S2, P5S9). Group IV comprised of 6 genotypes including 2 genotypes of population 1 (P1S5, P1S6), 2 from population 2 (P2S3, P2S6) and 2 from Population 4 (P4S7, P4S10). Group V were comprised of 6 genotypes including only 1 from population 3 (P3S3), 2 from population 4 (P4S4 and P4S9) while 3 from population 5 (P5S3, P5S4 and P5S6). Group VI cluster was found unique including only 1 genotypes from Population 3 (P3S4) which indicated that the populations 1, 2, 4 and 5 were related comprising of similar genotypes while population 3 was found to be distinct and unique having only one genotype.

Clustering of Turmeric populations collected from different regions of Bannu including Mandan, Ismail Khel, Kila Raakh Sarkar, Michen Khel and Kaki. It was noticed that Un-weighed Pair Group Arithmetic Averages (UPGMA) on the basis of Euclidean distance clustered these into two groups including Group I and Group II. Group I comprised of four populations P1, P4, P5 and P2 collected from Ismail Khel, Michen Khel, Mandan and Kaki respectively (Fig. 7). Group I was comprised of three clusters including P1 (Ismail Khel) in cluster I, P4 (Michen Khel) and P5 (Mandan) in cluster II while P2 (Kaki) in cluster III while Group II was found unique as it comprised of only one population P3 form Raakh Sarkar. Most of Turmeric populations, in the present study was found related having similarity matrix ranged from 89.2 to 100% as shown in Table 7. By comparing P1 with other populations, it was found highly related with P5 (98.3) followed by P4 (97.8) while it was found least related with P3 (89.9) of Group II. In comparison of P2 with other Turmeric populations, it was found highly related with P5 (95.8). P3 of Group II was found highly related with P2 (100) while it was least related with P4 (90.9) followed by P5 (91.0). Similarly P4 of Group 1 was found highly related with P2, P3 and P4 having similarity matrix 100% while it was least related with P5 (98.6). The analysis showed that Raakh sarkar turmeric genotypes are different from the Turmeric populations of other four regions of Bannu.

Clustering of Turmeric populations using UPGMA on the basis of Euclidean distance were analyzed in two groups including Group I and Group II. Group I comprised of four populations P1, P4, P5 and P2 collected from Ismail Khel, Michen Khel, Mandan and Kaki respectively. Group I comprised of three clusters including P1 (Ismail Khel) in cluster I, P4 (Michen Khel) and P5 (Mandan) in cluster II while P2 (Kaki) in cluster III. Similarly, Group II was observed as unique as it comprised of only one population P3 form Raakh Sarkar.

Most of Turmeric populations, in the present study was found related having similarity matrix ranged from 89.2 to 100% as shown in Table 7. By comparing P1 with other populations, it was found highly related with P5 (98.3) followed by P4 (97.8) while it was found least related with P3 (89.9) of Group II. In comparison of P2 with other Turmeric populations, it was found highly related with P5 (95.8). Similarly, P3 of Group II was found highly related with P2 (100) while it was least related with P4 (90.9) followed by P5 (91.0). In the same way, P4 of Group 1 was found highly related with P2, P3 and P4 having similarity matrix 100% while it was least related with P5 (98.6).

Thaikert & Yingyong (2009) find out antioxidant activity and genetic diversity in *Curcuma* species by using RAPD markers. They clustered all the accession into four groups. The accessions of turmeric population were mixed together and placed into several groups. Their results from cluster analysis did not show any distinct relationship with their region. The mixture of collection from different location in cluster may be due the movement of planting material throughout the country.

It was concluded that RAPDs marker evaluated in the present study were not highly efficient for fingerprinting of Turmeric geneotypes. Our investigation did not efficiently reveal the ability of RAPD markers to differentiate turmeric genotypes into their populations.

Table 7. Genetic distance matrix of five turmeric populations of District Bannu.

	P1	P2	P3	P4
P2	97.0			
P3	89.9	89.2		
P4	97.8	94.9	90.9	
P5	98.3	95.8	91.0	98.6

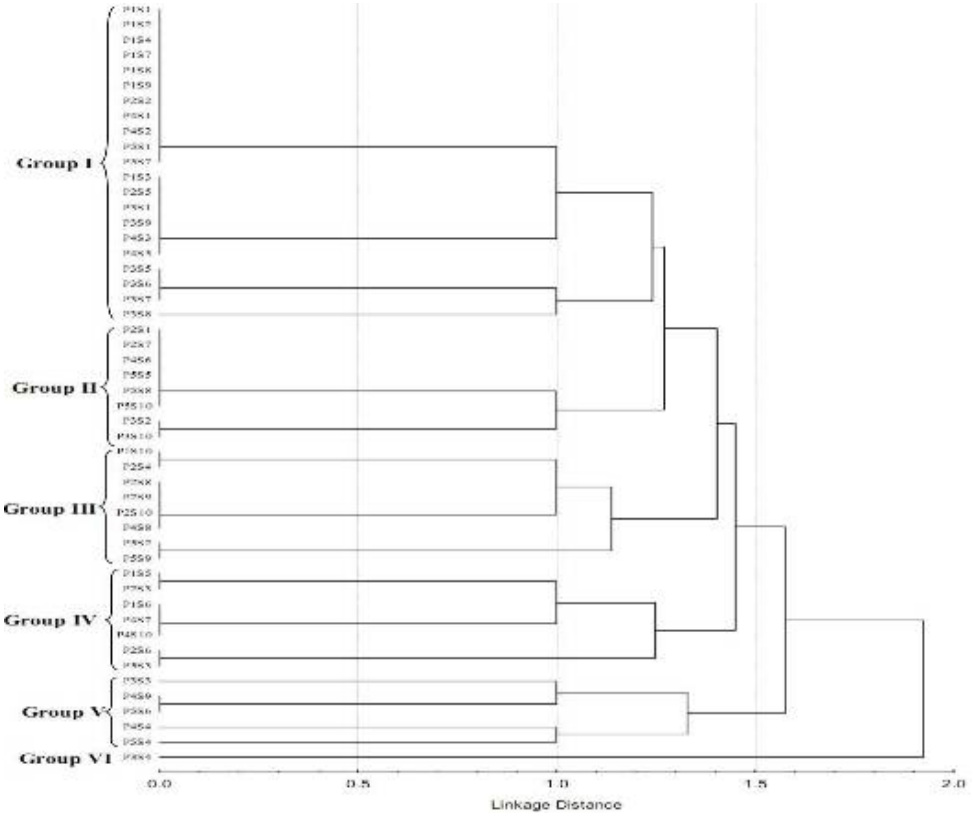

Fig. 6. Cluster analysis of different turmeric genotypes using un-weighed pair group arithmetic averages.

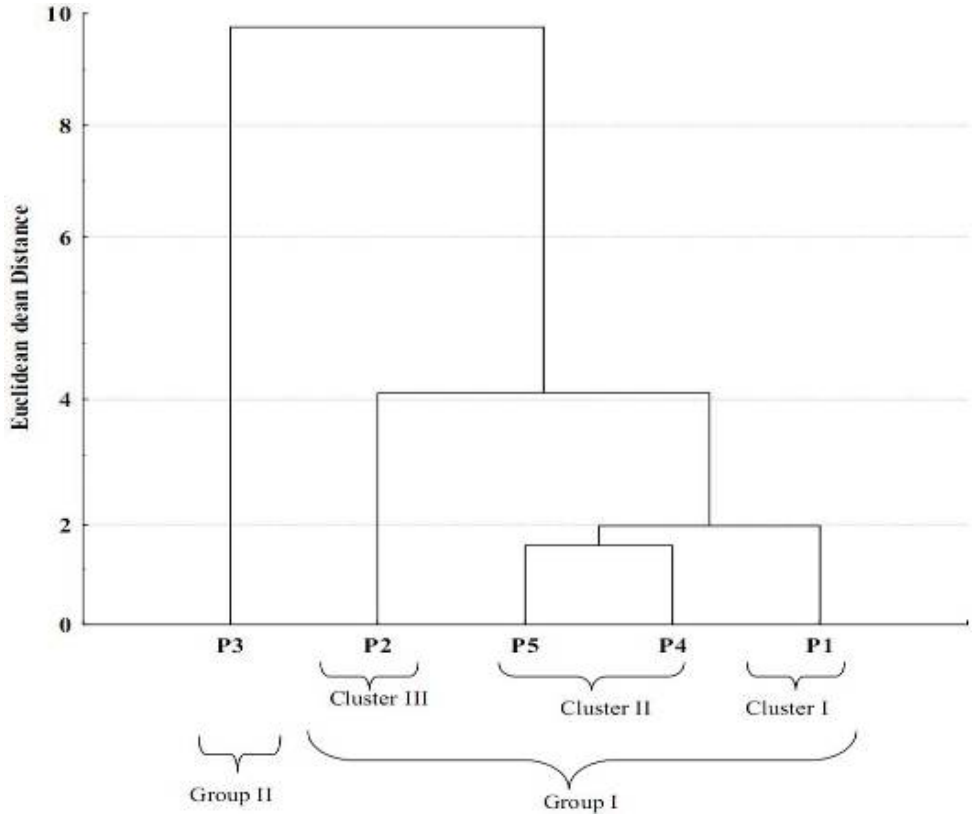

Fig. 7. Cluster analysis of different turmeric populations using Un-weighed Pair Group Arithmetic Averages (UPGMA).

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