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GENETIC DIVERGENCE AMONG *CURCUMA LONGA* L. (TURMERIC) GERMPLASM FROM PAKISTAN USING RAPD MARKERS

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ABSTRACT

Curcuma longa L. is a vegetatively-propagated crop which is known as spice crop and has valid medicinal importance due to many biological active compounds. Conventional breeding of turmeric is difficult and often limited to germplasm selection. Genetic divergence among turmeric (*C. longa* L.) genotypes collected from different ecological regions of Pakistan including Kasur, Changa manga, Faisalabad and Bannu was developed using Randomly Amplified Polymorphic DNA (RAPD) markers. Fifteen decamer primers generated 125 RAPD fragments, of which 91 bands were polymorphic with 72.22 % polymorphism. The number of amplified bands ranged from 3 (OPT-23) to 11 (OPT-11) with the size of amplicons varied from 200 bp (OPT-09) to 2850 bp (OPT-05). Similarity coefficient across 25 genotypes of turmeric started from 0.00 to 0.76. Dendrogram was generated through UPGMA and clustered 25 genotypes into four groups. Cluster analysis differentiated Bannu genotype as a separate group while Kasur, Changa manga and Faisalabad genotypes were closely related to each other. RAPD analysis can be helpful to select the different parents for genetic breeding programmes in future and to increase the essential oil and curcumin contents of turmeric for the fulfillments of demands of pharmaceutical industries.

Key words: Turmeric genotypes, genetic variation, Bannu, Polymerase chain reaction and Zingiberaceae.

INTRODUCTION

Curcuma longa (syn. *C. domestica*) is herbaceous plant of *Zingiberaceae* family and comprises about 70 species (Jan *et al*. 2012; Labban, 2014). It is native to tropical South Asia and maximum genetic diversity is found in India and Thailand with at least forty species in each area, followed by Berma, Bangladesh, Indonesia and Vietnam (Jan *et al*. 2011).India is the major producer and exporter of turmeric all over the world (Archana *et al*. 2013; Paul *et al*. 2016). In Pakistan it is mainly cultivated in the districts of Lahore, Kasur, Sialkot and Mandi Bahauddin in Punjab province and Bannu, Haripur and Pubbi areas in province Khyber Pakhtunkhwa (Jan *et al*. 2011; Khan *et al*. 2013). The district Kasur contributes more than eighty percent of the country production with 30569 metric tons annual production from 3157 ha (Anwar *et al*. 2012).

Curcuma longa (turmeric) is currently considered a potential source of new medicines for a variety of ailments due to the presence of curcuminoids (Corcolon, *et al*. 2015). Turmeric oleoresin contained 30-55 % of curcuminoids pigments and 15-25 % of volatile oil. The aroma of turmeric is due to its volatile oil while its yellow color is due to curcuminoid contents present in rhizome. Its rhizome powder has long been applied as coloring agent, spice, cosmetic and medicinal agent in Eastern and Asian cultures (Nasirujjaman *et al*. 2005; Tilak *et al*. 2004). Various monoterpenoids and sesquiterpenoids such as ar-turmerone, α- turmerone and curloneare the major part of essential oil of turmeric (Naz *et al.* 2010a and 2011). Curcumin makes up approximately 90% of the curcuminoid content in turmeric (Labban, 2014) which has potential of antispasmodic, antibacterial, antioxidant, anti-parasitic, anti-fungal, anti-carcinogenic and anti-inflammatory activities(Fan *et al*. 2013; Naz *et al*. 2010b; Baatout *et al*. 2004; Polasa *et al*. 1991). Leaves and rhizome of *C. longa* are also used as bio-fertilizer (Nayak and Naik 2006).

C. longa is a sterile and triploid species (2n = 3x = 63) that is vegetatively-propagated through its rhizomes (Islam *et al*. 2004). Since hybridization is difficult many times, genetic improvement is restricted to selection of germplasms and mutation breeding in few cases (Ravindran *et al*. 2007). Viable seeds can be only produced under specific conditions, which enable recombination breeding by selection of open-pollinated progeny and hybridization (Sasikumar, 2005). Recently, other alternatives have been successfully applied for example mutation breeding, somaclonal variations, genetic engineering and induction of polyploidy (Shirgurkar *et al*. 2006).

Information about genetic variability among species is very essential for breeding techniques and plant genetic resource conservation (Thaikert and Paisooksantivatana, 2009). Random amplified polymorphic DNA (RAPD) analysis in junction with polymerase chain reaction (PCR) is one of the techniques which use a single arbitrary sequence of 10 base oligonucleotides (Welsh and McClelland, 1990; Williams

et al. 1990) to generate DNA fragments that can be used as genetic markers to measure the extent of genetic diversity among individuals between and within species. Pinheiro *et al*. (2003) were the first who evaluated the genetic divergence in twenty accessions of *C. longa* using RAPD markers. Then Cintra *et al*. (2005) showed low genetic variability within accessions of *C. longa* from Brazil. Since knowledge about genetic divergence in the *C. longa* germplasm is mandatory to enhance the development of genetic resources and the efficiency of selection in breeding programs, the present investigation was undertaken to determine the genetic variation among accessions of *C. longa* from Pakistan using the RAPD technique.

MATERIALS AND METHODS

Plant materials: Different genotypes of *Curcuma longa* were collected from different ecological zones of Pakistan such as Kasur, Changa manga, Faisalabad and Bannu. Eight samples of turmeric were collected from of Kasur, five samples from Changa manga, five samples from Faisalabad and seven samples from Bannu population. The Sampling was done from October to December 2012. All samples were coded based on their origin and geographical descriptions with environmental parameters are presented in Table 1.

Table 1.List of samples of turmeric genotypes with their area of collection, genotypic codes and environmental parameters.

Collection Area	Province of collection	Genotype code	Latitude	Longitude	Temperature ºF		Average rainfall (mm)
					Min	Max	
District Kasur	Punjab	K1–K8	31º 5′ N	74º 24′ E	84	99	612.0
Changa manga	Punjab	CH1–CH5	31º 4′ N	73º 58′ E	91.4	116.6	663.9
District Faisalabad	Punjab	F1–F5	31º 25′ N	73º 4′ E	71.1	91.8	479
District Bannu	Khyber Pakhtunkhwa	B1–B7	32º 59′ N	70º 36′ E	61.5	69.3	416.0

Genomic DNA extraction: Fresh leaves of *C. longa* were used to extract genomic DNA by modified method of Doyle and Doyle (1990). In sterilized pestle and mortar about 0.1 gm of turmeric leaves samples were crushed using liquid nitrogen to get frozen powder. After that preheated extraction buffer (CTAB2%,Tris base100Mm, 20mM EDTA, and 1.4M NaCl with 1.0% mercaptoethanol) was added to frozen powder and eppendorf tubes were incubated at 65ºC for half an hour. An emulsion was formed by proper mixing of chloroform/isoamyl alcohol (24:1). All eppendorfs were spinned at 9000 rpm for 10 minutes. The supernatant solution was taken and again chloroform/isoamylalcohol was mixed and eppendorfs were spinned for ten minutes at 9000 rpm. The top aqueous phase was taken into new tube. For precipitation of DNA, chilled 2-propanol was poured, tubes were spinned for 5 minutes at 9000 rpm to get pellet and the supernatant solution was not needed. The ethanol was used for washing of pellet was washed and then air dried pellet was dissolved in 0.1x TE (1.0 mMTris base (pH 8.0) and 0.1 mM EDTA-pH 8.0) buffer or dH_2O. DNA dilutions were made and stored at 4ºC till further use.

Quantification of genomic DNA: The quantification of genomic DNA was possible through horizontal gel electrophoresis and 0.8% agarose gel in 1X TAE (Tris acetate EDTA) with ladder was utilized. In a jar, 0.8 gram agarose powder (Roth product) was weighed and 98ml of 1X TAE was added. Agarose was dissolved completely on heating and allowed to cool at room temperature. Ethidium bromide (0.2µg/ml) was mixed into the melted agarose gel and polymerized after half an hour at room temperature. Gel loading samples were prepared by taking 1µL of genomic DNA in an eppendorf and 2µL of 6X loading dye (Vivantis, UK product) was added. All the samples were loaded into the wells of submarine gel while in first well DNA ladder (Vivantis, UK product 3000bp) was loaded. The power supply was turned on and gel was started running at 110V for at least 35-45 minutes. The DNA started migration towards the positive (red) electrode and the movement of loading dye was seen with naked eyes. Then gel was documented using Dolphin gel documenting system to visualize the DNA bands. The size of each band was compared with DNA ladder.

RAPD analysis: Thirty five decamer oligonucleotides primers were applied for analyzing polymorphism. Polymerase chain reaction was done in volume of 15µl (12µl master mix + 3µl template genomic DNA) containing 10X PCR buffer (100 mMTris-HCl; ph 9.1, KCl 500 mM), $MgCl_2$ 1.5 mM, 200 µM each of dNTPs (Deoxynucleotide triphosphate), 0.2 µM of primer (Invitrogen USA), 0.5U Taq DNA polymerase, 20 ng of DNA (template genomic) and the volume was raised upto 15µl with double distilled deionized water. This used protocol was modified method of Williams *et al*., (1990).

Polymerase Chain Reaction (PCR): RAPD-PCR based amplification was carried out in thermal cycler (Primus-96 wells). It was programmed to first cycle of 4 minutes at 94ºC for initial denaturation of template DNA. This

was followed by 45 cycles with three steps. First step was template DNA denaturation at 94 ºC for 30 seconds, second and third steps were annealing of primer at 36ºCfor 1 min and extension of primer at 72ºC for 1:30 minutes respectively. Final extension was done for 7 minutes at 68ºC for the complete polymerization and all amplified products were stored at 4.0ºC. The PCR amplified products were resolved on 0.8% agarose gel (prepared in 1X TAE buffer) after adding 2.0µl loading dye which binds to amplified DNA. The results of each primer were repeated three times to check reproducibility of DNA bands. After electrophoresis the DNA band sizes were compared with DNA ladder acts as marker under UV light of gel documenting system (Wealtec Sparks, USA) and photographed.

Data analysis: Agarose gel photographs were compared with each other by calculating the presence and absence of DNA fragments. The presence of DNA fragments were replaced with "1" and absence were denoted by "0" in amplified profiles. The molecular size of amplicons was determined with a 3000bp marker (Vivantis UK product). Similarity matrix was developed by analyzing amplified profiles for the evaluation of genetic relation between various genotypes of turmeric. Corresponding dendrogram was formed by analyzing similarity coefficients with a cluster analysis using an Un-Weighted Pair Group Method with Arithmetic Averages (UPGMA) through NTSYS-pc(Numerical Taxonomy and Multivariate Analysis System version 2.01) by computerized software (Roulf, 2002).

RESULTS AND DISCUSSION

The genetic diversity and relationship among 25 turmeric genotypes collected from different populations of Pakistan were evaluated using RAPD markers. The number of total bands generated by each primer, the number of polymorphic fragments and the polymorphism percentage generated by each primer are given in Table 2. The amplification profiles generated by primers across 25 genotypes were shown in Fig. 1. A total of 15 decamer random primers were selected after screening 25 primers and produced 125 reproducible and scorable amplified bands out of which 34 were monomorphic and 91were polymorphic. The molecular size of bands ranged from 200 bp (OPT-09) to 2850 bp (OPT-05). It is evident from table that total amplified bands were highest (11) for OPT-11 where as OPT-23 produced lowest number of bands (3). The percentage of polymorphism varied from a highest of 84.61 % for OPT-11 to a lowest of 50.00 % by OPT-18.Thaikert and Paisooksantivatana, (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) utilized 10 RAPD primers for *C. longa* genotypes from different ecological zones of Pakistan and produced 95 amplified bands, of which 92 bands were polymorphic and percentage of polymorphism was 96.84%. The size of amplified fragments ranged from 200bp to 3640 bp. The polymorphism percentage varied from highest (100%) for 6 random primers to lowest of 66.67% by OPA-15. Then Khan *et al*., (2013) reported that polymorphism of turmeric genotypes collected from different regions of Bannu in Pakistan was 33.96% with primer OPA-03 while in the present study; polymorphism percentage of the turmeric genotypes was 84.61% with primer OPT-11. Nayak *et al*., (2006) also used rapid primers to characterize genetic variation among 17 cultivars of *C. longa* and the percentage of amplified fragments from35.6% in PTS51 to 98.6% in Acc31 was noted through twenty primers.

The RAPD is most widely used and popular technique because of its simplicity and rapidity and this method requires only a small quantity of DNA and have the potential to produce numerous polymorphisms (Williams *et al*. 1990). In the present study, average polymorphism percentage is 72.22 % among turmeric genotypes. This is in agreement with the observation of (Islam *et al*. 2007) that high percentage of polymorphism was present within the populations of *C. zedoaria*. Singh *et al*., (2012) used 11 RAPD primers and reported that 60 genotypes generated 94 amplified bands of which 75 were polymorphic with an average of 6.83 polymorphic bands per primer and showed 91.4% polymorphism. According to Nayak *et al*., (2006) this high level of polymorphism might be because of intra-specific variations among the turmeric cultivars. Thaikert and Paisooksantivatana, (2009) also suggested that high level of genetic divergence can exist within species of *C. longa*. Jan *et al*., 2011 suggested that high genetic variation in population of turmeric was possibly because of a wide range of ecological conditions in Pakistan. Genetic divergence through RAPD analysis have also been reported in many other plants also such as citrus (Naz *et al*., 2014), paw paw (Huang *et al*., 2003) tomato (Naz *et al*., 2013) and in chrysanthemum species (Naz *et al*., 2015).

In the present investigation, similarity matrix was utilized to develop the level of genetic relatedness between Kasur, Changa manga, Faisalabad and Bannu genotypes. The genetic distances between the Kasur, Faisalabad and Changa manga genotypes were comparatively lower than Bannu genotype. One pair of Bannu genotype B6 and B7 were closest genotypes with highest similarity index of 0.76 while four pairs of genotypes K1 and CH2, F1 and F2, K3 and CH4 and F4 and F5 with similarity index of 0.68 were the closest genotypes belonging to Kasur, Changa manga and Faisalabad. Yu and Nguyen, (1994) reported that IRAT 13 and IAC25 genotypes of rice were highly related by similarity coefficient of 0.79. It is clear from our results

that Bannu genotypes were placed in separate group and Kasur, Changa manga and Faisalabad genotypes were closely related and indicating highest genetic variation among population of *C. longa* in Pakistan. Jan *et al*., (2011) supported our results that Bannu and Kasur genotypes were placed at high genetic distances.

Cluster analysis placed many genotypes similar to each other belonging to different locations and indicating more genetic similarity. On the basis of Euclidean distance UPGMA (Un weighted Pair Group Arithmetic Averages) cluster was divided into two cluster I and II. According to the dendrogram, cluster I consisted of K1, K2, K3 K5, F1, F2, F3, CH2, CH3, CH4, CH5, B1, B2, B3, B4, B5, B6 and B7 belonging to Kasur, Faisalabad, Changa manga and Bannu genotypes whereas Cluster II had seven genotypes (K4, K6, K7, K8, F4, F5 and CH1). Cluster I gave high level of genetic variation among Kasur, Faisalabad, Changa manga and Bannu genotypes. Cluster I was next sub divided into two sub clusters. One Sub cluster I comprised of K1, K2, K3 K5, F1, F2, F3, CH2, CH3, CH4, B1, B2, B3, B4, B5, B6 and B7 collected from different populations while Sub cluster II had only one genotype CH5. In our investigation, cluster analysis showed genetic variations between genotypes collected from different ecological zones of Pakistan. It is evident from dendrogram that genotypes B1, B2, B3, B4, B5, B6 and B7 were genetically more similar with each other as compared to other tested genotypes. Our results are in accordance to the findings of (Jan *et al*., 2011) who reported that Bannu genotypes were grouped in one cluster and it might be due to have same ecological conditions as well as same ancestors. Genotypes F1 and F2 were closely related and similarly K1, CH2 and K3, CH4 and F4 and F5 were showing similar genetic relatedness collected from different populations of Pakistan. RAPD markers can be utilized to differentiate variation between different populations. Information related to genetic relationships among genotypes will be helpful for breeding programmes in future to choose genetically different parents of *C. longa* genotypes.

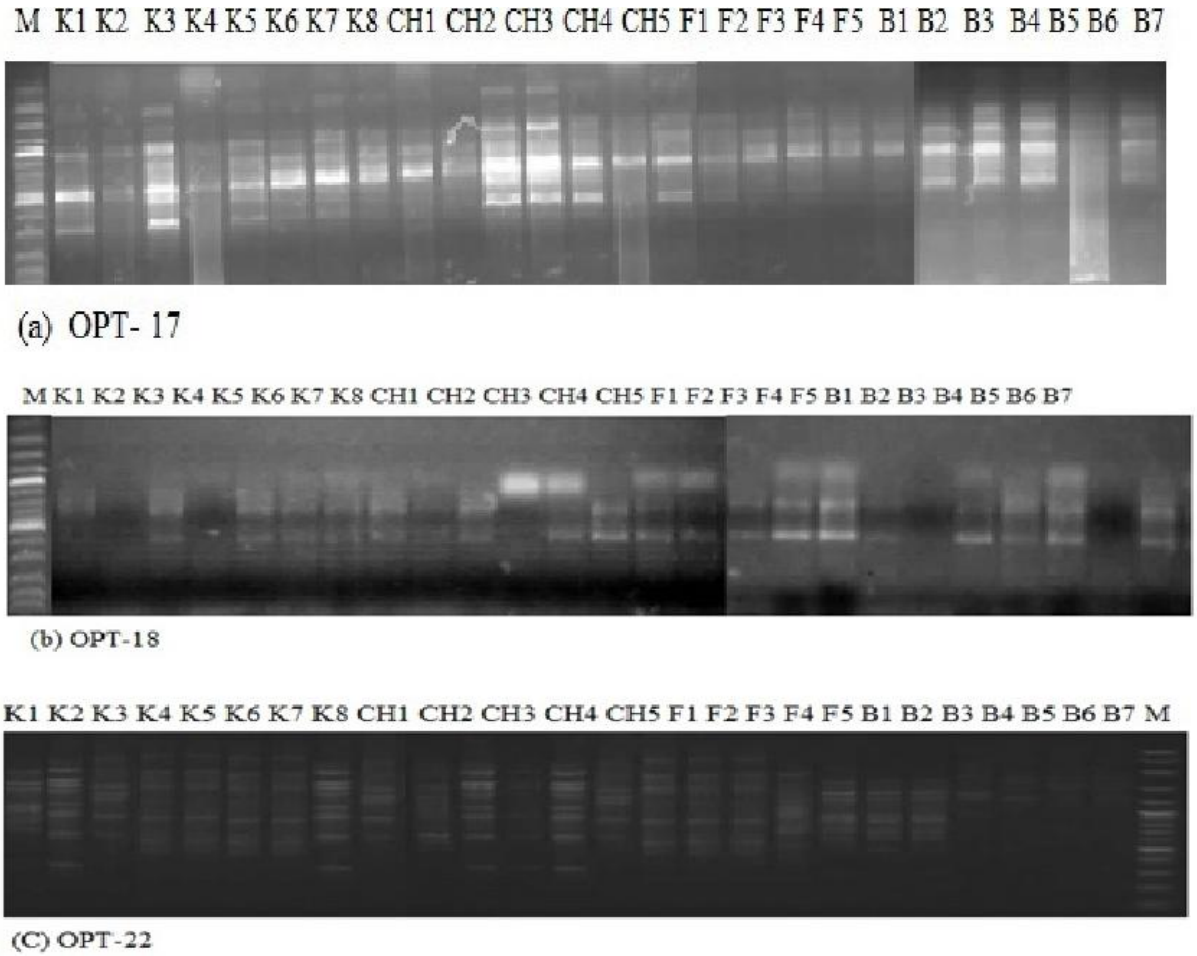


Fig.1: RAPD banding pattern of 25 genotypes of turmeric collected from four populations (Kasur, Changa manga, Faisalabad and Bannu) produced by rapid primers

Table 2. RAPD-PCR Primer sequences, total number of bands, polymorphic bands, percentages of polymorphism and size range of amplicons

Sr. No.	Primer codes	Primer sequence (5'……. 3')	Total bands	Polymorphic bands	Percentage polymorphism	Size range of amplicons (bp)
1.	OPT-01	GAAACGGGTC	10	8	80.00	300-1500
2.	OPT -03	CAATCGCCGT	8	5	62.50	800-2700
3.	OPT -05	CAAACGTCGG	9	6	66.66	1300-2850
4.	OPT -07	GTCGCCGTCA	7	5	71.42	450-2200
5.	OPT -08	AGGTGACCGT	6	4	66.66	400-1700
6.	OPT -09	CTCACCGTCC	11	9	81.81	200-2400
7.	OPT -11	GTCGCCGTCA	13	11	84.61	700-2600
8.	OPT -12	GTTGCGATCC	14	10	71.42	900-2300
9.	OPT -14	ACTTCGCCAC	7	4	57.14	1350-2600
10.	OPT -16	GAAACGGGTG	6	5	83.33	1300-2100
11.	OPT -17	CATCCGTGCT	8	6	75.00	1400-2000
12.	OPT -18	AATGGCGCAG	8	4	50.00	500-2700
13.	OPT -20	ACGCGCATGT	5	4	80.00	600-2400
14.	OPT -22	CTGACGTCAC	9	7	77.77	250-1600
15.	OPT -23	GACGGATCAG	4	3	75.00	700-2500
Total			**125**	**91**	**--**	**--**
Mean			**8.33**	**6.06**	**72.22**	**200-2850**

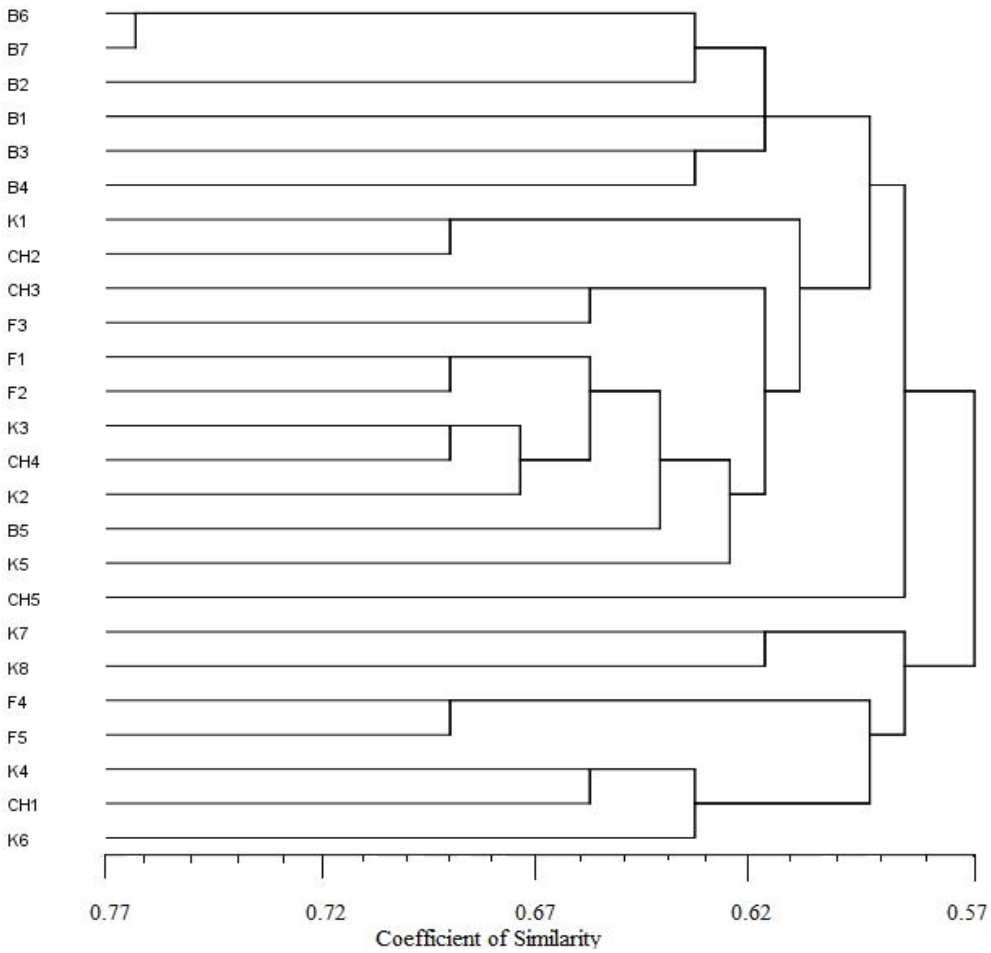


Fig.2:Dandrogram showing the genetic relationship (diversity) among 25 Pakistani turmeric genotypes, produced by UPGMA cluster analysis based on amplified bands generated by 15 random primers.

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