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Genetic diversity assessment of safflower (*Carthamus tinctorius* L.) genotypes through morphological and RAPD marker

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Abstract
The aim of the present study was to assess the fifteen Safflower (*Carthamus tinctorius* L.) genotypes for genetic diversity using morphological traits and RAPD markers. Nine morphological traits were studied and subjected to analysis of variance. Mean squares due to genotypes were highly significant as well as wide mean range performance was observed for plant height, seed yield and oil yield. Ward's cluster analysis based on morphological traits separated the accessions into two groups. RAPD analysis was carried out with 20 primers. They were screened but only 14 primers produced amplification. These 14 primers produced 74 bands, in which 64 were polymorphic bands. Average polymorphism was 86.49 per cent. Based on the RAPD markers, a dendrogram was constructed using the UPGMA method. The similarity coefficient ranged from 0.50 to 1.00 with an average of 0.75. Cluster analysis based on RAPD data separated the accession into four main groups. In general there was association between the dendrogram obtained by RAPD markers and morphological characteristics. The result obtained can also be used for further breeding purposes for characterization and identification of new genotypes.

Keywords: Safflower genotypes, RAPD, morphological traits, dendrogram

Introduction
Safflower (*Carthamus tinctorius* L.) is an important underutilized oil seed crop of the tropical countries, which belongs to the family *Compositae* having genome size 1.4 GB and 2n=24 chromosomes (Jhajharia, 2013) [13]. It is mainly cultivated for its seeds, which is used primarily for edible oil. Traditionally the crop was grown for its flowers, used for colouring and flavouring foods, making dyes and in medicine (Weiss, 2000). It is well known for its quality edible oil in view of higher proportion of linoleic and oleic acid content compared to other vegetable oils. The oil is semidrying in nature and being used in paints, textile and leather industries (Zhang, 1997) [27]. More recent studies has shown that transgenic safflower is used for the production of important pharmaceuticals of human use such as insulin (Carlsson, 2014) [5]. India is the largest producer of safflower in the world with an area of 3.00 lakh hectares, with the production of 1.89 lakh tonnes (2008-09). The area under safflower in India has been significantly decreasing from 8 lakh hectares in 1986 to 3.63 lakh hectares in 2006 (Anonymous, 2007). The trend is same in many safflower growing countries, although safflower is reported to be more remunerative than some traditionally grown crops under dry land conditions. However, due to an increasing demand for vegetable oil in human diet its production as an oil seed crop has received a great deal of attention, but its low oil content of 28-30 per cent and low yield of 600 kg/ha makes safflower a poor competitor.
Dramatic advances in molecular biology over the last few years have provided us with a range of new techniques for analysis of variation in plants at DNA level. DNA marker helps in analysis of species in relation to diversity. The precise cataloguing of species diversity by molecular markers has gained lot of attention in many crop plants. The molecular markers have been applied in limited in safflower. In the present study, cultivated as well as wild species of *Carthamus* were analysed by RAPD markers with a view, not only to assess their relative diversity but also to identify most suitable primers and markers for differentiating the species. RAPD markers are commonly used because they are quick and simple to analyse genetic diversity in several types of plant material such as natural populations, population in breeding programmes and germplasm collections. It does not require any sequential data because arbitrary DNA sequence is used as single primer which target unspecified genomic sequence to generate a genetic profile. The pattern of amplified sequence could be species or strain specific and constitute an identifying profile of the organism (Ferreira and Grattupaglia,

1996) [10]. RAPD markers are superior when simplicity and costs are considered (William *et al*., 1990) [26]. RAPD has been used in analysis of genetic distance in different plant species (Colombo *et al*., 2000; Chowdhury *et al*., 2017; Baruah *et al*., 2017) [7, 6, 4]. Another important application of molecular marker in genetic analysis involves improvement in the efficiency of conventional plant breeding by carrying out indirect selection through QTL. The predictive value of these markers used in Marker Assisted Selection (MAS) depends on their inherent repeatability, map position and linkage with economically important traits (quantitative and qualitative).

Materials and method

Present investigation was conducted on 15 safflower genotypes (Table 1). All the facilities related to present study were made available by the Department of Molecular Biology and Biotechnology and Department of Plant Breeding and Genetics, Rajasthan College of Agriculture, MPUAT, RCA, Udaipur.

Table 1: Fifteen diverse safflower genotypes having origin from Directorate of Oilseed Research, Hyderabad

Code No.	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15
Genotype	IVT-10-02	IVT-10-06	IVT-10-07	IVT-10-08	IVT-10-09	IVT-10-11	IVT-10-14	IVT-10-15	IVT-10-16	IVT-10-17	IVT-10-18	IVT-10-19	IVT-10-20	IVT-10-22	IVT-10-23

DNA isolation, quality testing and quantification

DNA extracted from the 15 safflower genotypes were compared by RAPD marker analysis. In this methodology, DNA was extracted from young leaves (3 weeks old plantlets) using CTAB method (Doyle and Doyle 1990) [8]. DNA was amplified by using random oligonucleotide primers in a DNA thermo cycler (Eppendorf). The amplified samples were separated on agarose gel electrophoresis (1.2%). The bands were scored for their presence or absence. DNA samples were quantified in nano- spectrophotometer. All chemicals used in DNA isolation and PCR technique were of analytical grade and purchased from Hi-media.

Optimization of PCR conditions and RAPD analysis

Random amplification of polymorphic DNA was done by using 15 primers obtained from Bangalore Genei Pvt. Ltd., Bangalore. PCR reaction was performed in a final volume of 20 µl containing 10X Reaction Buffer, 1 unit of Taq. DNA polymerase, 200 µM each of dNTPs mix, 0.5 µM/reaction of random primers and 50 ng of template DNA. The PCR was performed in PCR eppendorf thermocycler using the following cycling parameters: Initial denaturation step at 94ºC for 5 min followed by 40 cycles at 94ºC for 1 min, annealing at 36ºC for 1 min and extension at 72ºC for 1 min and final extension for 5 min at 72ºC. The amplified products were separated by 1.2 % agarose gel electrophoresis containing 0.5µ/ml ethidium bromide. The gel was viewed under UV transilluminator and photographed with the help of gel documentation system (Alpha DG DOC). A 100 bp DNA ladder was included in the gel as standard molecular weight marker. A set of 15 decanucleiotide RAPD primers were used for PCR amplification. The sequences of primers were selected from literature and purchased from Genei Pvt Ltd, Bangalore. Scoring of the RAPD products were done as- The presence of each band was scored as '1' and its absence as '0'. Faintly visible bands were not scored, but a major band corresponding to faint bands was considered for scoring. The scores (0 or 1) for each band obtained from photograph were entered in the form of a rectangular data matrix (qualitative data matrix). The pair-wise association coefficients were calculated from qualitative data matrix using Jaccard's similarity coefficient. Cluster analysis for the genetic distance was then carried out using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method. The genetic distances obtained from cluster analysis through UPGMA were used to construct the dendrogram, depicting the relationship of the genotypes using computer program NTSYSpc version 2.02 (Rohlf, 1997) [20].

Morphological Characters under Investigation

The experiment was laid out in randomized block design with three replications. Each entry was planted in 5 rows of 4 m length with a spacing of 45 x 20 cm. The recommended dose of fertilizer was applied at rate of 40 kg N2/ha, 40 kg P_2O_5/ha and 20 kg K2O/ha. All the recommended agronomic practices and plant protection measures were adopted to raise a healthy crop. Characters under investigation were [1] Days to 50 percent flowering, [2] Days to maturity, [3] Germination Percentage [4] Plant height (cm), [5] Number of effective capitula per plant, [6] Test Weight in gram (The weight of 100 seeds) [7] Seed yield (kg/ha), [8] Oil content (%) and [9] Oil Yield (kg/ha). The observations were recorded on 5 randomly selected plants for each entry (genotype), from all three replications. Oil content (%) and Oil Yield was calculated as:- two random samples of seeds were drawn from bulk harvest of 5 randomly selected plants under each replication and oil content was determined by the Soxhlet's method and average oil content (%) was calculated.

Result and discussion

Analysis of experimental design

The data of 9 morphological characters were subjected to analysis of variance for Randomized Block Design (RBD). The mean squares due to genotypes were significant for all the traits thereby indicating substantial amount of variability among the genotypes. The mean squares due to replications were also significant for number of pods per plant and branches per plant (Table 2).

Table 2: Analysis of Experimental Design

S. No.	Characters	Replication	Genotype	Error
	Degree of Freedom	(2)	(14)	(28)
1	Days to 50% Flowering	5.60	42.51	6.96
2	Days to Maturity	0.29	92.12	5.24
3	Germination Percentage (%)	2.22	126.03	64.13
4	Plant Height (cm)	0.98	360.01	32.09
5	Capitula per plant	4.02	28.90	16.26

6	Test Weight (g)	0.13	3.28	0.98
7	Seed Yield (kg/ha)	1548.95	1293797.50	58310.59
8	Oil Content (%)	0.027	6.872	1.221
9	Oil Yield (kg/ha)	279.76	107101.14	4887.67

A perusal of mean performance revealed that narrow mean range exhibited for the characters such as test weight (4.86-8.36) and oil content (26.09-31.88). Moderate mean range exhibited for characters such as days to 50% flowering (111.33-125.00), days to maturity (141.00-162.33), germination percentage (73.33-93.33), capitula per plant (25.33-36.33), while characters such as plant height (88.53-123.63 cm), seed yield (764.90-3126.68), oil yield (223.91-903.64) had wide mean range. The number of capitulas per plant, test weight, seed yield and oil yield had high value of variance which indicated that the diversity was present which could contribute to improvement of crop. Similar results were observed by Ali *et al.* (2007) [1] for a set of pea (*Pisum sativum* L.) germplasm showing the consistency of the traits in the germplasm. The average of oil content was 28.82 per cent and ranged from 26.09 to 31.88 per cent. The line IVT-10-08 (31.88%) was found most superior in oil content, followed by IVT-10-15 (30.87%). The line IVT-10-09 (26.09%) had minimum oil content. Similar results were reported by Pancholy *et al.* (1978). They analysed oil content in different groundnut cultivars and reported that per cent oil ranged from 46 to 52.6 per cent.

Classifying the Genotypes using Ward's Cluster Analysis (On the basis of Morphological Characters)

Ward's hierarchical cluster analysis carried out on the basis of 9 morphological characters. It was used to measure genetic distance between 15 safflower genotypes. Cluster analysis grouped the genotypes into two clusters, cluster I and II which were apart at 25 rescaled values. Cluster analysis was found useful by Salamati *et al.* (2011) in grouping safflower genotypes.

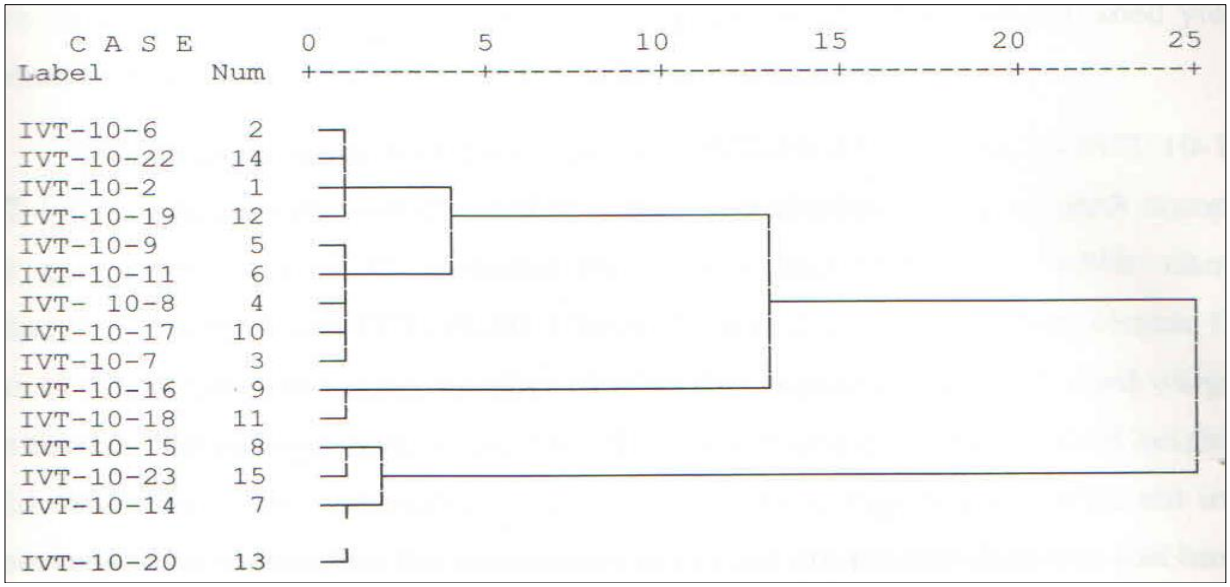

Fig 1: Dendroogram generated for 15 safflower genotypes using ward sclustering method based on morphologcical traits

Cluster I included eleven genotypes. This cluster was further subdivided into two sub clusters, A and B at 13 rescaled values. Sub cluster A included nine genotypes *viz.* IVT-10-6, IVT-10-22, IVT-10-2, IVT-10-19, IVT-10-9, IVT-10-11, IVT-10-8, IVT-10-17 and IVT-10-7. Sub cluster A further sub divided into two sub sub clusters A' and A''. A' included four genotypes *viz.* IVT-10-6, IVT-10-22, IVT-10-2 and IVT-10-19 and A'' included five genotypes *viz.* IVT-10-9, IVT-10-11, IVT-10-8, IVT-10-17 and IVT-10-7. A' and A'' were found 4 unit apart. The days to 50 per cent flowering, number of effective capitula per plant and germination percentage were similar for all these genotypes whereas plant height, seed weight, days to 50% maturity, seed yield, oil content and oil yield were significantly different in these genotypes.

Sub cluster B included two genotypes *viz.* IVT-10-16 and IVT-10-18. These genotypes were 1 unit away from each other. The days to 50 per cent flowering, number of effective capitula per plant, days to 50% maturity and germination percentage were similar for all these genotypes whereas plant height, seed weight, seed yield, oil content and oil yield were significantly different in these genotypes.

Cluster II included four genotypes *viz.* IVT-10-15, IVT-10-23, IVT-10-14 and IVT-10-20, which were sub divided into two sub clusters C and D each comprising two genotypes. Cluster C included IVT-10-15 and IVT-10-23 while cluster D included IVT-10-14 and IVT-10-20. Cluster C was 2 unit apart from cluster D. The days to 50 per cent flowering, number of effective capitula per plant, seed weight and germination percentage were at par for all these genotypes whereas plant height, seed yield, oil content, days to 50% maturity and oil yield were significantly different in these genotypes. This shows that the accessions in cluster are mostly identical and have less diversity. The results were in conformation with observation of Talebi *et al.* (2008) [23]. They studied genetic relationships among 36 accessions of chickpea. Cluster analysis based on morphological traits separated the accessions into 3 clusters.

Assessment of amplified fragments obtained from RAPD

All the 15 varieties of safflower cultivars were examined for DNA polymorphism using 20 decamer primers (OPERON) showing high (G+C) content. Out of 20 primers, 14 primers produced amplification whereas 6 primers *viz.* OPK-7,

RKAT-3, RKAT-4, RKAT-9, RKAT-10 and RKAT-14 did not show any amplification. Out of 14, 13 primers showed variable degree of polymorphism ranging from 60 per cent (OPK-9(A)) to 100 per cent (OPK-10, RKAT-1, RKAT-2, RKAT-5, RKAT-6, RKAT-7, RKAT-8, RKAT-12 and RKAT-13), whereas one primer *viz.* RKAT-11 did not show any polymorphism. Overall polymorphism was found to be 86.49 per cent. Similar results were reported by Reddy *et al.* (2004) in groundnut.

Fourteen primers on fifteen safflower genotypes generated 74 total bands out of which 64 were polymorphic. In given genotypes, for each evaluated primer produced 3 to 8 fragments and their size ranged between ~100 bp to ~2100 bp. The average number of bands per primer was found to be 5.3. The results were in the confirmation of observations by Amiri *et al.* (2001) [3]. They reported that amplified DNA fragment length ranged from ~300 bp to ~2400bp. Similar results were also reported by Javed *et al.* (2009) [12] in mustard. The maximum number of amplicons was produced by the primers RKAT-13 (74) which was followed by primer RKAT-2 (72). Band pattern with primer RKAT-13 is shown in figure 1. The minimum number of amplicons was produced by the primer RKAT-7 (24). Among all the primers tested, RKAT-2 and RKAT-12 proved to be the best primers as they produced 72 and 38 amplicons, respectively and 8 scorable bands in each, of which all were polymorphic. Average polymorphism was 100 per cent.

Similar results were reported by Pathak *et al*. (2010) [17]. They resulted that out of 10 decamer primers 5 primers generated a total of 49 amplicons out of which 39 were polymorphic and polymorphism ranged from 66.6 to 87.5 per cent. The results obtained were in conformity with the earlier reported by Reddy *et al*. (2004). Thus, it is opined that RAPD assays can be efficient in identifying DNA polymorphism provided suitable primers are used.

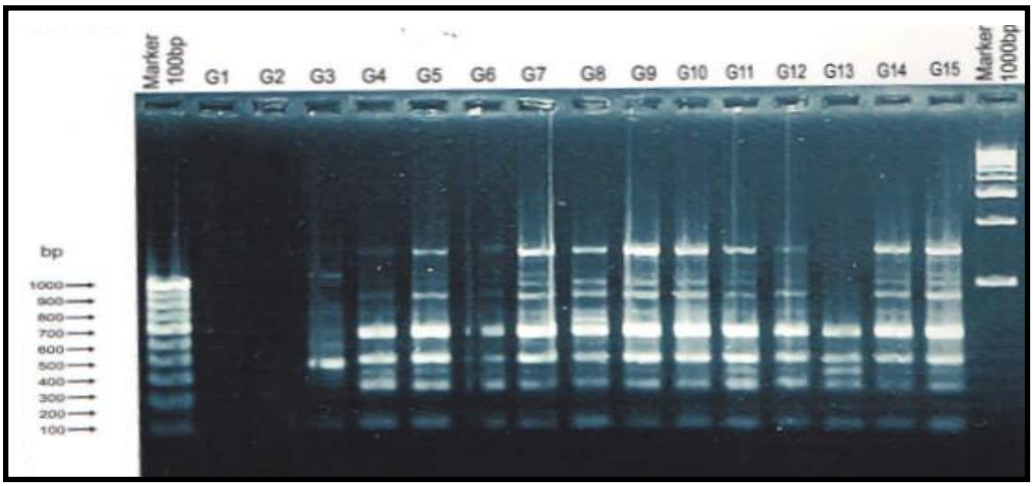

Fig 1: RAPD profile of safflower genotypes (G1-G15) generated with primer RKAT-13

The banding pattern generated and polymorphism reflected in RAPD was used to calculate the genetic similarity among the 15 safflower cultivars taken for the present study. Genetic similarity estimates based on RAPD banding patterns were calculated using method of Jaccard's coefficient analysis (Jaccard, 1908) [11]. The similarity coefficient matrix generated was subjected to algorithm UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and dendrogram was generated using NTSYSpc 2.02 program (Rohlf, 1997) [20]. The RAPD data were used to obtain a similarity matrix. The similarity coefficients for different genotypes were ranged from 0.50 to 1.00. The average similarity across all the genotypes was found to be 0.75 indicating a high level of genetic similarity among the genotypes. The results obtained were in accordance with the earlier reported by Dwivedi *et al*. (2001) [9].

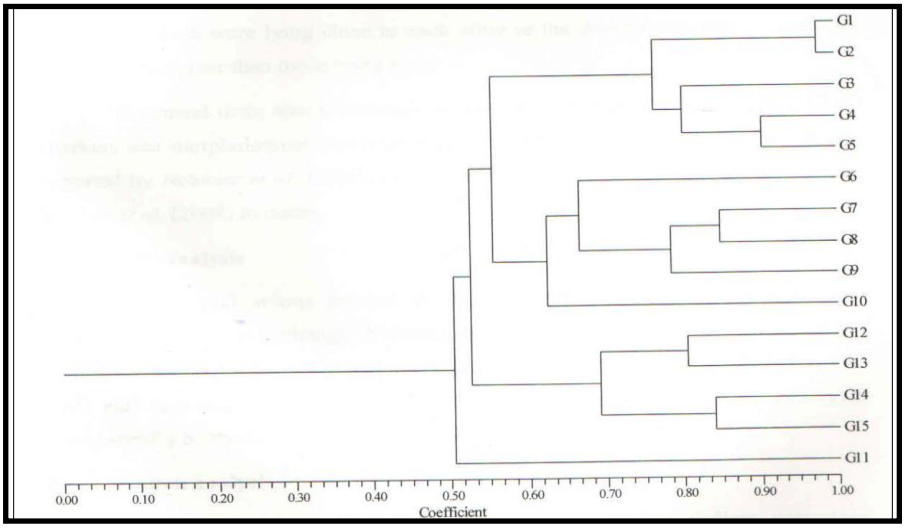

Fig 2: Dendrogram generated for 15 safflower genotypes using UPGMA cluster analysis based jaccard similarity coefficient

They selected 26 accession of groundnut to study molecular diversity using RAPD assay (8 primers). The polymorphism among primers ranged from 8.73 per cent (GN20) to 33.08 per cent (N4). The similarity coefficient ranged from 0.59 to 0.98 with an average of 0.86. Similar results were also reported by Kumari *et al*. (2009) [14].

The maximum similarity coefficient (0.98) was observed between IVT-10-2 and IVT-10-6, followed by IVT-10-8 and IVT-10-9 and IVT-10-14 and IVT-10-15 with the similarity of 0.90 and 0.85, respectively. The minimum similarity coefficient (0.37) was observed between IVT-10-6 and IVT-10-18. Similar results were obtained by Amadou *et al.* (2001) [2] and Shrivastava *et al*. (2008) [22].

A dendrogram was constructed using similarity matrix value as determined from RAPD data for 15 genotypes using UPGMA subprogramme of NTSYSpc programme (Figure 2). The dendrogram depicted the relationship among the 15 safflower genotypes and clearly divided them into 4 main clusters. Cluster I which was the major cluster included five genotypes *viz.* IVT-10-2, IVT-10-6, IVT-10-7, IVT-10-8 and IVT-10-9 at similarity coefficient of 0.77. This cluster was divided into two sub cluster, sub cluster A and B. Sub cluster A included two genotypes *viz.* IVT-10-2 and IVT-10-6 which were related to each other at similarity coefficient of 0.98 and morphologically also both were similar. Total 9 morphological characters were studied in which 2 *viz.* days to 50 per cent flowering and germination percentage were similar in these genotypes. Similar results were reported by Wang *et al.* (2011) [25]. They studied RAPD based genetic diversities and correlation with morphological characters in *Camellia* (*Theaceae*) cultivars in China. They found a correlation between cluster analyses based on molecular markers and flower color in the first group. Sub cluster B included three genotypes *viz.* IVT-10-7, IVT-10-8 and IVT-10-9 at similarity coefficient of 0.80 and morphologically also all were similar. Sub cluster B was further sub divided into two subgroups B` and B``. B` included one genotype IVT-10-7 which was joined with (IVT-10-8 and IVT-10-9) at similarity coefficient of 0.80. B`` consists of two genotypes IVT-10-8 and IVT-10-9 at similarity coefficient of 0.91. Out of 9 morphological characters, 3 *viz.* days to 50 per cent flowering, numbers of effective capitula per plant and germination percentage were similar in these genotypes. Cluster II also included five genotypes *viz.* IVT-10-11, IVT-10-14, IVT-10-15, IVT-10-16 and IVT-10-17 at similarity coefficient of 0.62 with cluster I. Cluster II divided into two subclusters, A and B. Sub cluster A was further sub divided into subgroups A' and A''. A' included only one genotype IVT-10-11 which was joined with A" (IVT-10-14, IVT-10-15 and IVT-10-16) at similarity coefficient 0.67. Second sub group A'' was again sub divided into two sub groups A_1'' and A_2''. A_1'' included two genotypes *viz.* IVT-10-14 and IVT-10-15 at similarity coefficient 0.84 and morphologically also both were similar, while A_2'' included only one genotype *i.e.* IVT-10-16 which was joined with A_1'' at similarity coefficient 0.79 but morphologically different from sub cluster A_1". Out of 9 morphological characters, 3 *viz.* days to 50% flowering, germination percentage and oil content were at par in these genotypes. Sub cluster B included only one genotype *i.e.* IVT-10-17 which joined with sub cluster A at similarity coefficient 0.62. Cluster III included four genotypes *viz.* IVT-10-19, IVT-10-20, IVT-10-22 and IVT-10-23 at similarity coefficient of 0.69. Cluster III joined cluster II at similarity coefficient of 0.54. Cluster III was divided into two sub cluster, A and B. Sub cluster A included two genotypes *i.e.* IVT-10-19 and IVT-10-20, with similarity coefficient of 0.80 but morphologically both were in different cluster. Out of 9 morphological characters, 4 *viz.* days to 50 per cent flowering, number of effective capitula per plant, seed weight and germination percentage were similar in these genotypes. Sub cluster B also included two genotypes IVT-10-22 and IVT-10-23 at similarity coefficient 0.83. Sub cluster A joined with sub cluster B at similarity coefficient of 0.69. Out of 9 morphological characters, 3 *viz.* number of effective capitula per plant, seed weight and germination percentage was similar in these genotypes. Cluster IV included only one genotype *i.e.* IVT-10-18 at similarity coefficient of 0.50 with cluster III. Therefore, the cluster tree, revealed a similar results about 15 genotypes. The association amongst different genotypes was presented in the form of dendrogram, the genotypes which were lying close to each other in the dendrogram were genetically closer to each other than those lying apart. In general there was association between the dendrogram obtained by RAPD markers and morphological characteristics. This was in accordance with the results reported by Nebauer *et al.* (2000) [15] in the genus *Digitalis*, Raza *et al.* (2018) [18] in Sunflower and Thakur *et al.* (2008) [24] in maize.

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