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Molecular characterization of African marigold (*Tagetes erecta*) genotypes using RAPD markers

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ABSTRACT

The present investigation was carried out to characterize 22 genotypes of marigold (*Tagetes erecta* L.) with RAPD (randomly amplified polymorphic DNA) markers. A total of 26 RAPD markers were utilized for assessment of genetic divergence in marigold which amplified a total of 241 amplicons, out of which 238 (98.80%) were found polymorphic. The average number of amplicon per primer was 9.27. The Jaccard's similarity coefficient ranged from 0.18 to 0.80. The mean values of polymorphic information content (PIC), resolving power (RP) and marker index (MI) of RAPD primers were 0.38, 8.36 and 3.44 respectively. Cluster analysis was done by unweighted pair group method for arithmetic average (UPGMA) and Principal Component analysis (PCA) was also done for the grouping of genotypes. The broad range of Jaccard's similarity coefficient observed for the genotypic data generated suggest the wide genetic base for marigold genotypes which will be effectively explored for future breeding programmes.

Key word: Genetic diversity, RAPD, *Tagetes erecta*, UPGMA

Marigold is one of the important flower crop belonging to the family Asteraceae. The genus *Tagetes* comprises approximately 50 species and among these, *Tagetes erecta* L.(African marigold), *T. patula* L. (French marigold) and *T. tenuifolia* Cav. (Striped marigold) are most common in cultivation due to their ornamental values (Soule 1996). In India, it is grown as one of the major loose flower crop which covers an area 55.89 ('000 ha) and production of 511.31 ('000 MT) (Anonymous 2014). The marigold flowers are always in great demand for garland making and decorative purposes at various kinds of religious and social ceremonies. Nowadays, marigold is also being utilized as a landscape plant due to its high ornamental values like varied shape, colour, form, size *etc*. Marigold is rich in essential oils so it is being utilized in high grade perfumes and cosmetic products. Moreover, the oil has also been reported to possess insect repellent properties. Marigold flowers are also considered as a highly rich source of carotenoids especially lutein and being used in poultry feed so as to intensify the yellow colour of egg yolk and broiler skin. Moreover, crop has the potential for its use in nutraceutical and pharmaceutical industries (Boonnoun *et al*. 2012).

The variability present in germplasm is prerequisite for the success of any breeding programme so arises the need to characterize the germplasm at morphological and molecular level. Characterization will provide thorough description of genotypes which will further be used in conservation and crop improvement programmes. Assessment of genetic diversity based on morphological markers has many disadvantages like most of the morphological traits are highly influenced by environmental conditions and moreover they are specific to development stages of the plant. Morphological characterization involves assessment of germplasm at different stages and moreover, it is not very reliable method as many of traits of interest have low heritability and are genetically complex too. In contrast, molecular markers based on DNA sequence polymorphism are independent of environmental conditions and show higher level of polymorphism. Molecular markers provide a quick and reliable method for the assessment of genetic relationships among the genotypes of any organism (Thormann *et al*. 1994). Among the different types of molecular markers, randomly amplified polymorphic DNAs (RAPDs) are useful for the assessment of genetic diversity owing to their simplicity and relatively low-cost as compared

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to other types of molecular markers (William *et al*. 1990, Rafalski and Tingey 1993). Furthermore, RAPD markers are easy to generate and allow the analysis of very small amount of DNA (Tingley and Tufo 1993). RAPD markers have been extensively used to distinguish intra-specific genetic variation in ornamental crops. This is because RAPD analysis does not require pre-existing knowledge about DNA sequence of the organism. Many crop species as well as ornamental plants have been characterized using RAPD technique. The genetic diversity exhibited among marigold genotypes has not yet been rigorously defined. Moreover, studies involving molecular markers are also limiting factor. Consequently, we used RAPD markers in marigold to: 1) discriminate among various genotypes; 2) characterize genetic relationships among and between genotypes.

MATERIALS AND METHODS

The planting material comprised of three male sterile lines, two varieties and 17 selections (Table 1) were grown and maintained at the Research Farm, Division of Floriculture and Landscaping, ICAR-Indian Agricultural Research Institute, New Delhi. The laboratory work was done at National Research Centre on Plant Biotechnology, Pusa Campus, New Delhi. Genomic DNA from young healthy leaves of the young marigold seedlings was extracted by CTAB (Cetyl Trimethyl Ammonium Bromide) method with minor modifications (Murray and Thomson 1980, Saghai-Maroof *et al.* 1984). Subsequent purification of crude genomic DNA was done and further quantified and assessed for quality by 0.8 % agarose gel electrophoresis. Aliquots for working samples were prepared by diluting a part of the original stock to final concentration 25 ng/µl.

A set of 26 RAPD primers (Table 2) were used for PCR amplification in 22 marigold genotypes. PCR amplification conditions were: initial extended step of DNA denaturation at 94°C for 4 minutes followed by 40 cycles of denaturation at 94°C for 1 minute, primer annealing at 37°C for 1 minute and elongation at 72°C for 2 minutes, followed by an elongation step at 72°C for 10 minutes. The reaction products were mixed with 2 µl of 10 X loading dye. The amplification products were separated on 1.5% agarose gels. Electrophoresis was carried out at 120 V for 2.5 hours using horizontal gel electrophoresis system. A 1 kb DNA ladder was run alongside the amplified products to determine the approximate band size of PCR product. The resolved amplification products were visualized by using UV-Transilluminator and photographs were taken with Gel Documentation system.

All amplifications were repeated twice and only reproducible bands were considered for analysis. Each

Table 1 Details of marigold genotypes used in genetic diversity analysis

Genotypes	Abbreviated	Plant height	Flower type	Flower form	Flower diameter	Flower colour
Male Sterile line -5	MS-5	Medium	Apetalous	*	Small	Yellow
Male Sterile line-7	MS-7	Medium	Apetalous	*	Small	Light Orange
Male Sterile line-8	MS-8	Medium	Apetalous	*	Small	Orange
Pusa Narangi Gainda	PNG	Tall	Petalous	Double	Large	Orange
Pusa Basanti Gainda	PBG	Tall	Petalous	Double	Large	Yellow
African Selection 1	Af.Sel.1	Small	Petalous	Double	Large	Orange
African Selection 2	Af.Sel.2	Medium	Petalous	Double	Large	Yellow
African Selection 3	Af.Sel.3	Medium	Petalous	Double	Medium	Yellow
African Selection 4	Af.Sel.4	Medium	Petalous	Double	Large	Light Orange
African Selection 5	Af.Sel.5	Small	Petalous	Double	Large	Orange
African Selection 6	Af.Sel.6	Tall	Petalous	Double	Large	Yellow
African Selection 7	Af.Sel.7	Medium	Petalous	Double	Medium	Yellow
African Selection 8	Af.Sel.8	Medium	Petalous	Double	Large	Yellow Orange
African Selection 9	Af.Sel.9	Medium	Petalous	Double	Large	Yellow
African Selection 10	Af.Sel.10	Medium	Petalous	Double	Large	Orange
African Selection 11	Af.Sel.11	Medium	Petalous	Double	Medium	Yellow Orange
African Selection 12	Af.Sel.12	Medium	Petalous	Double	Medium	Yellow
African Selection 13	Af.Sel.13	Medium	Petalous	Double	Large	Yellow
African Selection 14	Af.Sel.14	Tall	Petalous	Double	Large	Yellow
African Selection 15	Af.Sel.15	Medium	Petalous	Double	Medium	Yellow
African Selection 16	Af.Sel.16	Small	Petalous	Double	Large	Orange
African Selection 17	Af.Sel.17	Medium	Petalous	Double	Medium	Yellow Orange

*Only stigmas were present in the form of brush called as "Femina". Plant height (Small: 0- 45 cm; medium: 46-60 cm; Tall: >60 cm); Flower diameter (Small: 0-3.5cm; Medium: 3.6-5.0 cm; large: >5.0 cm)

Table 2 Details of banding pattern and discriminative statistics of RAPD markers used in genetic diversity analysis in marigold

Primer	Sequence (5'-3')	TB	PB	P(%)	PIC (SD)	RP	MI
OPA-4	AATCGGGCTG	12	12	100	0.37 (±0.26)	10.18	4.42
OPA-2	TGCCGAGCTG	1	1	100	0.5 (±0)	1.09	0.5
OPA-11	CAATCGCCGT	8	8	100	0.31 (±0.14)	7.91	2.48
OPA-14	TCTGTGCTGG	5	5	100	0.42 (±0.07)	6.18	2.12
RAPD-1	CAGGCCCTTC	10	8	80	0.27 (±0.19)	13.27	1.71
RAPD-2	GTGCCTAACC	15	15	100	0.44 (±0.09)	13	6.63
RAPD-3	GAAACGGGTG	14	14	100	0.41 (±0.06)	17.55	5.8
RAPD-4	TGGTGACTGA	13	13	100	0.44 (±0.06)	12.27	5.75
RAPD-5	TGCCGAGCTG	11	11	100	0.37 (±0.16)	6.36	4.03
RAPD-9	AGGTGACCGT	12	12	100	0.39 (±0.09)	12.64	4.73
RAPD-10	GGGTAAAGCC	14	14	100	0.36 (±0.14)	13.82	5.1
RAPD-13	TTTGGGCCCC	3	3	100	0.39 (±0.14)	2.73	1.18
RAPD-14	AGCCCCCAAG	14	14	100	0.41 (±0.1)	14.55	5.7
RAPD-18	CTGCCACGAG	8	8	100	0.27 (±0.16)	6.36	2.15
RAPD-19	GAGCAAGGCA	9	9	100	0.35 (±0.11)	6.36	3.18
RAPD-20	CTACGATGCC	11	11	100	0.36 (±0.11)	8.09	3.99
RAPD-12	CTGCTTCGAG	8	8	100	0.34 (±0.19)	4.91	2.74
RAPD-11	CCATGCGGAG	12	12	100	0.39 (±0.09)	7	4.62
RAPD-7	GGTGCACGTT	8	8	100	0.43 (±0.05)	5.09	3.41
RAPD-8	TGGTCACAGA	6	6	100	0.31 (±0.2)	3	1.86
RAPD-17	GGTGTTTGCC	10	10	100	0.38 (±0.14)	12.64	3.83
RAPD-15	CTGTCTGTGG	11	11	100	0.44 (±0.08)	8.82	4.86
RAPD-6	ACGTAGCGTC	6	6	100	0.33 (±0.19)	3.55	2
RAPD-22	TTACCCCGCT	2	2	100	0.29 (±0.29)	1	0.58
RAPD-16	ACTGGGTCGG	9	8	89	0.31 (±0.18)	11.91	2.19
RAPD-21	CCAGTCCCAA	9	9	100	0.43 (±0.09)	7.18	3.87
Total	26 primers	241	238	98.8	0.38 (±0.14)	8.36	3.44

band or amplification product was considered as a marker. The bands were scored as 1 for present or 0 for absent and missing data was denoted by (9) for construction of binary matrix. The discriminatory power of markers was estimated by three parameters, i.e. polymorphic information content (PIC), Resolving power (Rp), marker index (MI). The polymorphic information content (PIC) for each RAPD was calculated as proposed by Roldan-Ruiz *et al*. (2000). Resolving power (Rp) of primers was calculated according to Prevost and Wilkinson (1999). The third parameter used was the marker index (MI) as proposed by Powell *et al*. (1996). Jaccard's coefficient (Jaccard 1908) was used to calculate the genetic distance among cultivars as revealed by RAPD markers. Cluster analysis was done by unweighted pair group method for arithmetic average (UPGMA) and a dendrogram was generated using Numerical Taxonomy and Multivariate Analysis System Version 2.0 (Rohlf 2000). The reliability of UPGMA tree was further confirmed by bootstrap analysis using 1000 permutations. The Jaccard's similarity matrix was also subjected to principal coordinate analysis (PCA).

RESULTS AND DISCUSSION

The flower crops represent a wide range of diversity and success of any breeding programme depends on available genetic diversity in the germplasm. PCR-based molecular markers can play an important role in the analysis of genetic diversity analysis for further flower crop improvement programmes. The DNA profiles proved to be an useful evidence for the determination of purity of many cultivars and also in prevention of infringements of property rights in many horticultural crop species (De Riek 2001). Molecular markers play an important role in research areas, viz. gene mapping, population genetics, molecular evolutionary genetical studies *etc.* The RAPD (William *et al.* 1990) markers have been widely used in many flower crops for estimation of various kinds of genetic studies (Werlemark *et al.* 1999,Yamagishi *et al.* 2002, Nimusa *et al.* 2003).

RAPD polymorphism

A total of 241 reproducible amplicons generated by 26 RAPD primers, out of which 238 were found polymorphic (Table 1). The percentage of polymorphism ranged from 80% to 100% with an average of 98.80%. The polymorphism observed here was comparatively higher than previously observed in related species like 7% in *Tagetes minuta* (Daroker *et al.* 2000), 70% in *Tagetes patula* (Daroker *et al.* 2000), 57.92% (Namita *et al*. 2013) and 84% (Mor *et al.* 2008) in marigold, 69.51% in sunflower (Isaacs *et al*. 2003), 89.7% in *Chrysanthemum morifolium* (Xu *et al*. 2006) and 83.33% in *Chrysanthemum grandiflorum* (Kumar *et al*. 2014). In the present study, a wide range for the number of amplicons per primer was observed, i.e. primer OPA-2 amplified single amplicon, whereas highest 15 amplicon were scored for the primer RAPD-2. The other primers which showed high amplicons were OPA-4 (12), RAPD 3(14), RAPD- 4 (13), RAPD-9 (12), RAPD-10 (14), RAPD-14 (14) and RAPD-11(12). The average number of amplicons per primer was 9.27 which were almost similar to 9.75 in rose (Panwar 2015), 9.41 in chrysanthemum (Kumar *et al*. 2014) and 10.69 in marigold (Namita *et al*. 2013). The amplification profiles showing DNA banding pattern with RAPD markers were shown in Fig 1.

Studies about the discriminatory power of RAPD primers have been carried out in marigold to a less extent and in present study, average polymorphism information content (PIC) for RAPD markers was recorded as 0.38. The PIC value ranged from 0.27 (RAPD-1 and RAPD-18) to 0.5 (OPA-2). Other primers having higher PIC values were OPA-14 (0.42), RAPD-2 (0.44), RAPD-3 (0.41), RAPD-4 (0.44), RAPD-14 (0.41), RAPD-7 (0.43), RAPD-15 (0.44) and RAPD-21 (0.43). These results are in confirmation with Namita *et al.* (2013) and reported PIC value of 0.191 using RAPD markers in marigold. Kumar *et al.* (2014) also reported PIC value of 0.32 in chrysanthemum.

The RP ranged from 1 (RAPD-22) to 17.55 (RAPD-3) with average of 8.36. Other primers having higher RP values were RAPD-1(13.27), RAPD-2 (13.00), RAPD-4 (12.27), RAPD-9 (12.64), RAPD-10 (13.82), RAPD-14 (14.55) and RAPD-17 (12.64). Jhang *et al*. (2010) reported Rp (5.56) in carrot for RAPD markers. Namita *et al.* (2013) reported Rp of 3.48 in marigold. The MI ranged from 0.50 (OPA-2) to 6.63 (RAPD-2) with an average of 3.44. The other primers recorded higher MI values were RAPD-3 (5.80), RAPD-4(5.75), RAPD-10(5.10) and RAPD-14(5.7). Namita *et al*. (2013) obtained marker index 2.36 in marigold. The Jaccard's similarity coefficients for 231 combinations of 22 marigold genotypes based on 26 RAPD markers were computed. The similarity coefficients ranged from 0.18 to 0.80.

Cluster analysis based on RAPD analysis

The phylogenetic tree developed by clustering based on similarity matrix among the 22 marigold genotypes assessed using genotyping of 26 RAPD primers is shown in Fig 2. All genotypes were grouped in two major clusters assorted at similarity coefficient of 0.31. Consequently, the largest cluster including twenty genotypes were denoted as cluster I and the smaller with only two genotypes denoted as cluster II. Further the cluster I found to be grouped into two sub-clusters, cluster IA and IB. The sub cluster IA consisted of 18 genotypes namely Af.Sel.1, Af.Sel.7, Af.Sel.4, Af.Sel.9, Af.Sel.2, Af.Sel.6, Af.Sel.5, Af.Sel.11, Af.Sel.15, Af.Sel.12, Af.Sel.8, Af.Sel.16, Af.Sel.14, Af.Sel.3, Af.Sel.13, Af.Sel.10, Af.Sel.17 and Pusa Narangi Gainda and it further bifurcated into two sub clusters. However, sub cluster IB consisted of two genotypes like Pusa Basanti Gainda and MS-5. Cluster II consisted of two genotypes namely MS-7 and MS-8 and these findings are in accordance with the findings of Namita *et al*. (2008) in marigold. The association amongst different genotypes was presented in the form of dendrogram prepared using rescaled distances. The resemblances coefficient between the two genotypes is the value at which their branches join. The dendrogram also showed the relative magnitude of resemblance among different clusters.

Principal component analysis

The PCA analysis helps in identifying the most relevant characters and presents them in more interpretable and more visualized dimensions through linear combination of variables that account for most of the variation present in the original set of variables. It was revealed that MS-7 and MS-8 are closely related, whereas MS-5 is different from both MS-7 and MS-8. The results of principal component analysis were in close agreement with the results obtained by hierarchical cluster analysis (Dendrogram). Cultivars belonging to a common cluster have fallen nearer to each other and vice-versa, thereby confirming the results of

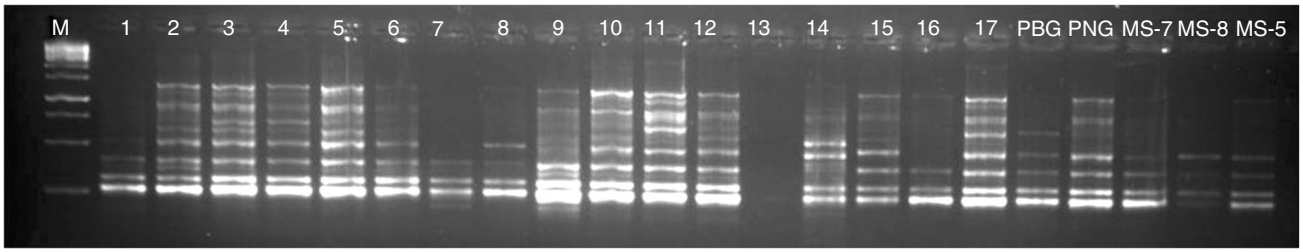

Fig 1 PCR amplification profiles of 22 marigold genotypes with RAPD-1 primer. Lane M represents 1kb marker [M, 1 kb ladder, Lane 1-17 represent different selection of marigold, 18-Pusa Narangi Gainda, 19-Pusa Basanti Gainda, 20-22 represents MS-7, MS-8 and MS-5]

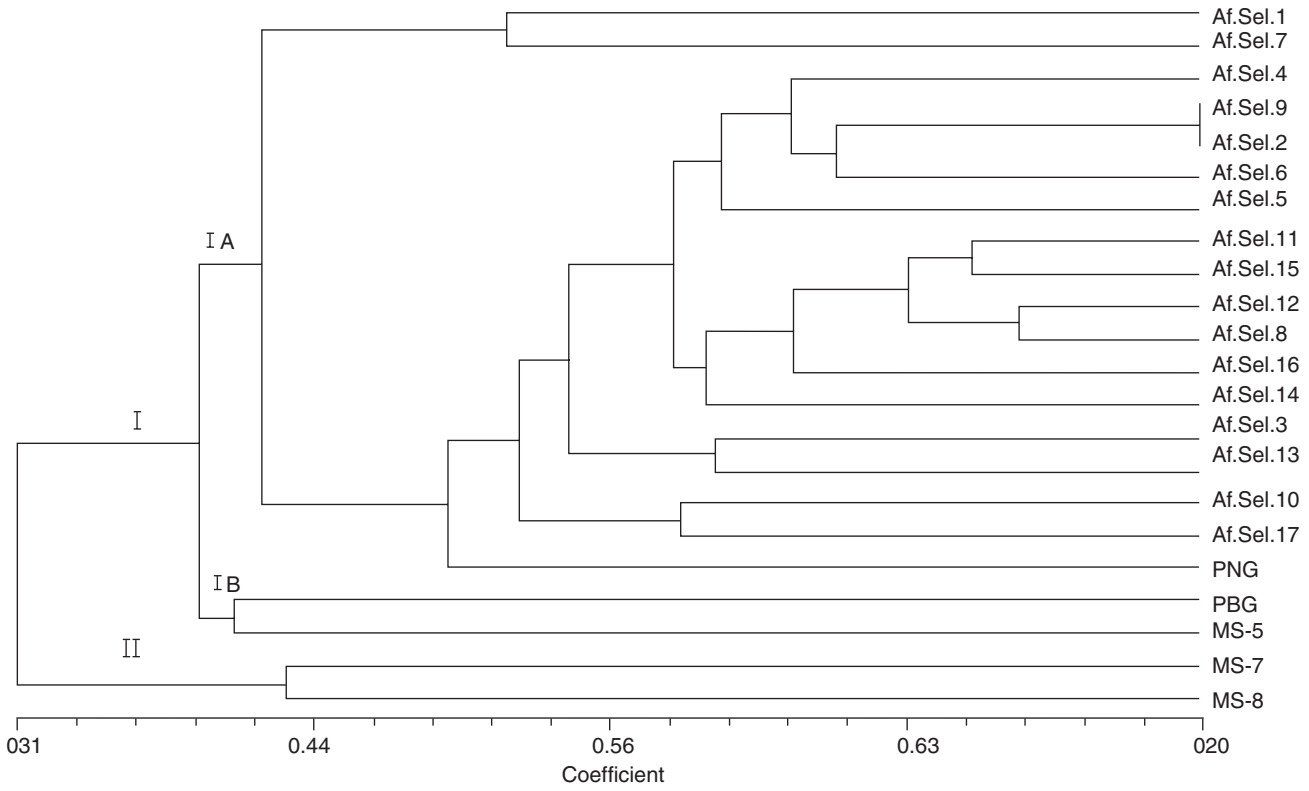

Fig 2 Dendrogram depicting genetic relationship among 22 marigold genotypes based on RAPD markers using Jaccards' similarity coefficient

dendrogram.

The genetically diverse population can serve as gene pool for many novel traits which will be utilizing in future crop improvement programmes for the development of marigold varieties with novel traits.

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