Evaluation of Genetic Diversity Analysis of Marigold (*Tagetes* Spp.) Accessions using RAPD Markers under Tropical Conditions in India

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Abstract

Twenty six (26) genotypes of marigold (Tagetes spp.) at the three different locations were characterized through electrophoresis using RAPD markers under South-Gujarat agro-climatic conditions of India. Out of 150 primers of RAPD screened, 13 RAPD primers were used to generate polymorphism for all genotypes yielding 136 bands, of which 47 were polymorphic with 34.34 % polymorphism with an average of 3.61 polymorphic fragments per primer. The dendrogram obtained from UPGMA cluster analysis of Jaccard's similarity values ranged from 0.11 to 0.69 for RAPD primers. Based on above markers, the genotypes were grouped mainly into two major clusters I and II with four sub-clusters viz., clusters I-A, cluster II-B, II-C and II-D including genotypes 3,3,10 and 10 respectively.

The wide range of Jaccard's similarity coefficient obtained for the genotypic data of three different locations suggests the wide genetic base for marigold genotypes and location based effect on phenotype and genotype characters. These can be effectively explored for the future breeding programs.

Keywords: Marigold, Molecular marker, RAPD, Genetic diversity.

Introduction

Marigold (*Tagetes* spp.) is one of the most important traditional flower crops grown in India, owing to its ornamental and industrial uses. It is grown for landscaping and occupies an ever increasing demand in medicinal and industrial sector. In India, about 3,06,280 ha. area is under floriculture with annual production of 16, 99,420 metric tonnes of loose flowers and 6,92,840 metric tonne of cut flowers in 2016-17[3]. Marigold is grown in India around the area of 66,130 ha. with a production of 6,03,180 metric tonnes[2]. It is widely grown for its loose flowers used for religious offerings and making garlands during social functions and as a bedding plant in landscape gardening. French marigold is suited for pots, rockery, edging, hanging baskets and window boxes. Of late, the extraction of carotenoides from petals for industrial uses raised the importance of this crop and increased the area under its cultivation. The carotenoides are mixed with poultry feed to intensify yellow colour of egg yolk. Lutein accounts for 80 to 90 % (w/w) of total carotenoides and is the major source of pigments for colouring food stuffs in place of synthetic colours. It has medicinal properties also and is used in the preparation of lotion, creams and tablets intended for the treatment of eye ailments.

Further, dietary carotenoides have the properties to treat cancer and other photo sensitive diseases in humans. Among all species, *Tagetes minuta* gives highest oil yield of 0.8 and 1.0 percent from leaves and flowers respectively. The genus, Tagetes comprises more than 50 species of annual and perennial herbs belonging to the family Asteraceae which also includes of other cultivated flowers like ageratum, aster, chrysanthemum, cornflower, cosmos, dahlia, daisy, gaillardia, sunflower etc.

In plant breeding genetic diversity plays a very important role as it helps in selecting the suitable parents for hybridization program resulting in superior hybrids and desirable recombinants[21]. It is also necessary to screen and develop stable genotypes which perform more or less uniform under varying environmental conditions. Thus, knowledge of genotype x environment interactions helps the breeder to select high yielding and more adaptable varieties or hybrids.

Morphological and molecular markers represent a potential goldmine of important information which can be applied as an efficient tool in crop improvement programmes. The major limitation of phenotypic markers is that many cultivars cannot be readily distinguished by morphological indices, particularly if they are close relatives and moreover, they show environment dependent expression.

However, molecular markers show genetic differences on a more detailed level and without interferences from environmental factors and involves techniques that provide fast results detailing genetic diversity[28]. Different types of molecular markers are available according to their potential to detect differences between individuals, their cost, facilities required, consistency and replication of results.

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However, the combined use of different markers can provide more reliable information about genetic diversity when compared to the use of only one marker. The errors or problems presented by a certain marker could be minimized using other markers[24,28]. The discovery that PCR with random primers can be used to amplify a set of randomly distributed loci in any genome facilitated the development of genetic markers for a variety of purposes[29,30].

RAPD (random amplified polymorphic DNA) markers have been extensively used to distinguish intra-specific genetic variation in ornamental crops. Looking at the importance and commercial potential, there is an urgent need to conserve and characterize the available variability and its evaluation to identify potential genotypes which would result in further improvement and to develop cultivar for specific uses.

Material and Methods

Marigold genotypes: The present investigation of genetic diversity analysis in different genotypes of marigold (*tagetes* spp.) using RAPD markers was conducted at Department of Plant Molecular Biology and Biotechnology, ASPEE College of Horticulture and Forestry, Navsari Agricultural University, Navsari, Gujarat, India. Field experiment was carried out at three different locations viz. Floriculture Research Farm (Dist. Navsari), Regional Rice Research Station (Vyara, Dist.Tapi), Hill millet Research Station, Waghai (Dist. The Dang) of Navsari Agricultural University, South Gujarat, India during Rabi season following general agriculture practices (GAP) as shown in table 1. In total, 26 genotypes of different marigold (Table 2) were evaluated in this study and their growth locations are shown in fig. 1.

Table 1
List of South Gujarat locations where different genotypes were grown

Environment	Location	Longitude	Latitude	Altitude (MSL) (m)	Type of soil	Season
E_1	Navsari	75^0 95'E	20° 95'N	10.0	Deep black	*Rabi*
E_2	Vyara	73^0 20'E	20^0 10'N	69.0	Deep black	
E_3	Waghai	73^0 50' E	20^0 77' N	107.0	Red	

Table 2
List of different marigold germplasm samples assessed for genetic variability using

S.N.	Genotype	Plant height	Flower colour	Flower type	Supplier/procured from
1	Pusa Narangi Gainda	Tall	Orange	Double	Indian Agriculture Research Institute , New Delhi
2	Summer Sugat	Medium	Orange	Double	Plantsman's Seeds, Patiala, Punjab
3	Namdhari African Orange	Tall	Orange	Double	Namdhari Seeds Pvt. Ltd., Karnataka
4	Hawaii Orange	Medium	Orange	Double	Plantsman's Seeds, Patiala, Punjab
5	Swati Orange	Tall	Orange	Double	DevkishanjiVaktaji& Sons, Gujarat
6	Indus Orange Bunch	Tall	Orange	Double	Indus Seeds, Bangalore, Karnataka
7	Local Selection 1	Tall	Orange	Double	Bardoli, Tapi, Gujarat
8	Suvarna Orange	Tall	Orange	Double	Suvarna Hybrid Seeds Pvt. Ltd., Bangalore
9	F_1 White Dwarf	Small	Creamy white	Semi double	Plantsman's Seeds, Patiala, Punjab
10	Local Selection 2	Tall	Orange	Double	Dahegam, Gandhinagar, Gujarat
11	Local Selection 3	Tall	Orange	Double	Jalalpore, Navsari, Gujarat
12	Local Selection 4	Tall	Orange	Double	Jalalpore, Navsari, Gujarat
13	Local Selection 5	Tall	Orange	Double	Vansda, Navsari, Gujarat
14	Local Selection 6	Tall	Orange	Double	NAU, Navsari, Gujarat
15	Local Selection 7	Tall	Orange	Double	NAU, Navsari, Gujarat
16	Local Selection 8	Tall	Orange	Double	Onjal, Navsari, Gujarat
17	Local Selection 9	Tall	Orange	Double	Dahegam, Gandhinagar, Gujarat
18	Inca Gold	Small	Orange	Double	Syngenta India Ltd., Pune
19	Local Selection 10	Tall	Orange	Double	Padra, Vadodara, Gujarat
20	Local Selection 11	Tall	Orange	Double	Kotha, Navsari, Gujarat
21	Local Selection 12	Tall	Orange	Double	Kotha, Navsari, Gujarat
22	Local Selection 13	Tall	Orange	Double	Kotha, Dist.- Navsari, Gujarat
23	Inca Yellow	Small	Yellow	Double	Syngenta India Ltd., Pune
24	Local Selection 14	Tall	Orange	Double	Kotha, Navsari, Gujarat
25	Sonata Orange	Small	Orange	Double	Plantsman's Seeds, Patiala, Punjab
26	Local Selection 15	Tall	Orange	Double	Dahegam, Gandhinagar, Gujarat

Random Amplified Polymorphic DNA markers

Fig. 1: Field locations of three different South Gujarat regions (a) Floriculture Research Farm, Navsari (b) Regional Rice Research Station, Vyara, Dist. Tapi and (c) Hill millet Research Station, Waghai, Dist. The Dang in India

DNA extraction: Total genomic DNA of 26 marigold genotypes was extracted from 500 mg of young leaf samples using the CTAB (Cetyl Trimethyl Ammonium Bromide) method of Doyle and Doyle[7] with a little modification. The leaves were first ground to a fine powder in liquid nitrogen using autoclaved and pre-chilled mortar and pestle. It was then transferred into a pre-warmed extraction buffer (1.5 M NaCl; 100 mM Tris-HCl with pH 8; 40 mM EDTA; 1 % PVP; 3 % CTAB and 1 % β- mercaptoethanol) and incubated at 65 °C for 1 hour. Equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added, mixed well by gentle inversion and centrifuged. The supernatant was transferred to a fresh tube and DNA was precipitated by adding 0.6 volume of ice-cold isopropanol. After centrifugation, the pellet was washed in 70 % ethanol, dried and dissolved in TE buffer. RNA was removed by RNase treatment. DNA was quantified using UV-spectrophotometer and diluted to 50-60 ng/µl and used in PCR.

PCR conditions and electrophoresis: Polymerase chain reaction (PCR) for RAPD analysis was performed in 20 µl volume containing 1x PCR buffer (Banglore Genei), 1.5 mM

$MgCl_2$, 0.2 mM dNTPs, 0.1 µM of primer, 50 ng of genomic DNA and 1 U Taq DNA polymerase (Banglore Genei). The reaction mixture was placed on a DNA thermal cycler (Biometra). RAPD program was performed as 1 cycle of 94 °C or 5 minutes and 40 cycles of 94 °C for 1 minute, 38 °C for 1 minute and 72 °C for 2 minutes, then, a final extension step at 72 °C for 8 minutes. PCR products were electrophoresed on 1.5 % (w/v) agarose gels, in 0.5X TBE Buffer (pH 8.3 ± 0.1) at 80 V for 1 hour and then stained with ethidium bromide (0.5 µg/ml). Gels with amplified fragments were visualized and photographed under UV light[29].

Scoring and data analysis: The DNA bands were scored for computer analysis on the basis of presence or absence. If a product was present in a genotype, it was designated "1", if absent, it was designated "0" after excluding irreproducible bands. Pair-wise comparisons of genotypes based on presence or absence of unique and shared polymorphic products were used to generate similarity coefficients based on similarity matching which were evaluated by calculating the Jaccard's similarity coefficient. The similarity matrix was subjected to the cluster analysis of unweighted pair group method with arithmetic averages (UPGMA) and a dendrogram was generated by using NTSYS-pc (Numerical Taxonomy and Multivariate analysis) version 2.2 software[23].

Results

Molecular analysis: The flower crops represent a wide range of diversity and success of any breeding program depends on available genetic diversity in the germplasm. PCR-based molecular markers can play vital role in the analysis of genetic diversity for further flower crop improvement programs. The DNA profiles proved to be a valuable evidence for the determination of purity of many cultivars and also in eradication of infringements of property rights in many horticultural crop species.

Molecular markers play key role in research areas *viz.* gene mapping, population genetics, molecular evolutionary genetical studies etc. The RAPD markers have been widely used in many flower crops for estimation of various kinds of genetic studies[15,17,25,33]. The RAPD profiles obtained using random primers varied considerably. The RAPD profiles obtained by various primer combinations are explained as follows:

RAPD banding patterns and dendrogram analysis of marigold germplasm: The data generated on the basis of RAPD markers were subjected to cluster analysis to understand their diversity and relatedness. A total of 136 reproducible amplicons were generated by 13 RAPD primers, out of which 47 were found polymorphic and 89 were monomorphic (Table 3). The percentage of polymorphism ranged from 11.11 % to 58.33 % with an average of 34.34 % as mentioned in table 3.

In the present study, a wide range for the number of amplicons per primer was observed i.e. highest 13 amplicons were scored for the primers OPF-12 and OPE-5 whereas lowest 8 amplicons were scored for the OPM-19. The other primers which showed amplicons were OPE-3 (10), OPE-10 (9), OPE-11 (10), OPF-13 (7), OPG-1 (9), OPG-3 (12), OPG-10 (11), OPG-12 (10), OPG-13 (12), OPM-12 (9), OPM-15 (10) and OPM-19 (8). The average number of amplicons per primer was 10.46. The PCR amplification profile was pictorially presented in fig. 2.

Table 3
Details of amplification obtained with different RAPD primers

S.N.	Name of primer	Primer sequence (5'-3')	No. of total bands	No. of polymorphic bands	No. of monomorphic bands	Polymorphism percentage (P %)	Total no. of bands amplified
1.	OPE-3	CCAGATGCAC	10	3	7	30.00	131
2.	OPE-5	TCAGGGAGGT	13	4	9	30.76	164
3.	OPE-10	CACCAGGTGA	9	3	6	33.33	126
4.	OPE-11	TTGGTACCCC	10	4	6	40.00	130
5.	OPF-12	ACGGTACCAG	13	3	10	23.07	165
6.	OPG-1	CTACGGAGGA	9	1	8	11.11	135
7.	OPG-3	GAGCCCTCCA	12	7	5	58.33	118
8.	OPG-10	AGGGCCGTCT	11	5	6	45.45	141
9.	OPG-12	CAGCTCACGA	10	4	6	40.00	129
10.	OPG-13	CTCTCCGCCA	12	4	8	33.33	146
11.	OPM-12	GGGACGTTGG	9	1	8	11.11	137
12.	OPM-15	GACCTACCAC	10	4	6	40.00	129
13.	OPM-19	CCTTCAGGCA	8	4	4	50.00	128
Total	13		136	47	89	34.34	1779

The Jaccard's similarity coefficients for 231 combinations of 26 marigold genotypes based on 26 RAPD markers were computed. The similarity coefficients ranged from 0.11 to 0.69. The phylogenetic tree developed by clustering based on similarity matrix among the 26 marigold genotypes assessed using genotyping of 13 RAPD primers is shown in fig. 3 and table 4. All genotypes were grouped in two major clusters assorted at similarity coefficient range 0.31. Consequently, the largest cluster including twenty three genotypes was denoted as cluster-II and the smaller with three genotypes denoted as cluster-I-A. Further, the cluster-II was found to be grouped into three sub-clusters, cluster II-B, II-C and II-D.

The cluster I-A consisted of 3 genotypes namely Indus Orange Bunch, local selection 1 and Suvarna Orange. While cluster II is divided into three sub-clusters, cluster II-B consisted of Namdhari African Orange, Local Selection 4 and Hawaii Orange; cluster II-C included Summer Sugat, local selection 6, local selection 10, local selection 15, local selection 3, local selection 11, local selection 14, local selection 13, local selection 5 and local selection 7 and cluster II-D involved Pusa Narangi Gainda, Swati Orange, F1 White Dwarf, local selection 2, local selection 9, Local Selection 12, local selection 8, Inca Gold, Sonata Orange and Inca Yellow.

Table 4
Clusters of marigold genotypes based on quantitative characters

Clusters	Sub-clusters	No. of genotypes	Marigold genotypes
I	A	3	Indus Orange Bunch, Local Selection 1, Suvarna Orange
II	B	3	Namdhari African Orange, Local Selection 4, Hawaii Orange
	C	10	Summer Sugat, Local Selection 6, Local Selection 10, Local Selection 15, Local Selection 3, Local Selection 11, Local Selection 14, Local Selection 13, Local Selection 5,Local Selection 7
	D	10	Pusa Narangi Gainda, Swati Orange, F_1 White Dwarf, Local Selection 2, Local Selection 9, Local Selection 12, Local Selection 8, Inca Gold, Sonata Orange, Inca Yellow

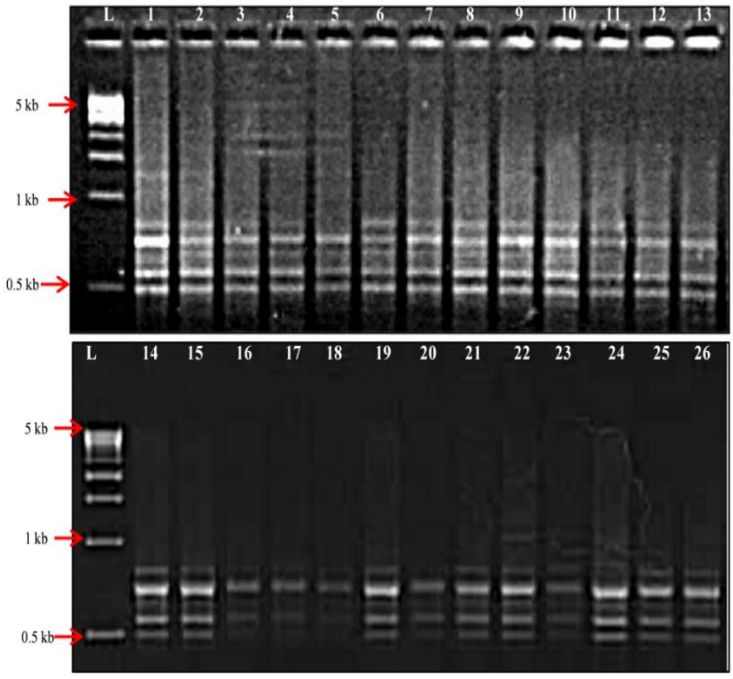

Fig. 2: RAPD amplification pattern of 26 marigold genotypes using RAPD primer OPG-1.L is the DNA marker (5kb), Lanes 1-26: 26 accessions of marigold that is Pusa Narangi Gainda, Summer Sugat, Namdhari African Orange, Hawaii Orange, Swati Orange, Indus Orange Bunch, Local Selection 1,Suvarna Orange, F1 White Dwarf, Local Selection 2, Local Selection 3, Local Selection 4, Local Selection 5,Local Selection 6, Local Selection 7, Local Selection 8, Local Selection 9, Inca Gold, Local Selection 10, Local Selection 11, Local Selection 12, Local Selection 13, Inca Yellow, Local Selection 14, Sonata Orange, Local Selection 15 respectively

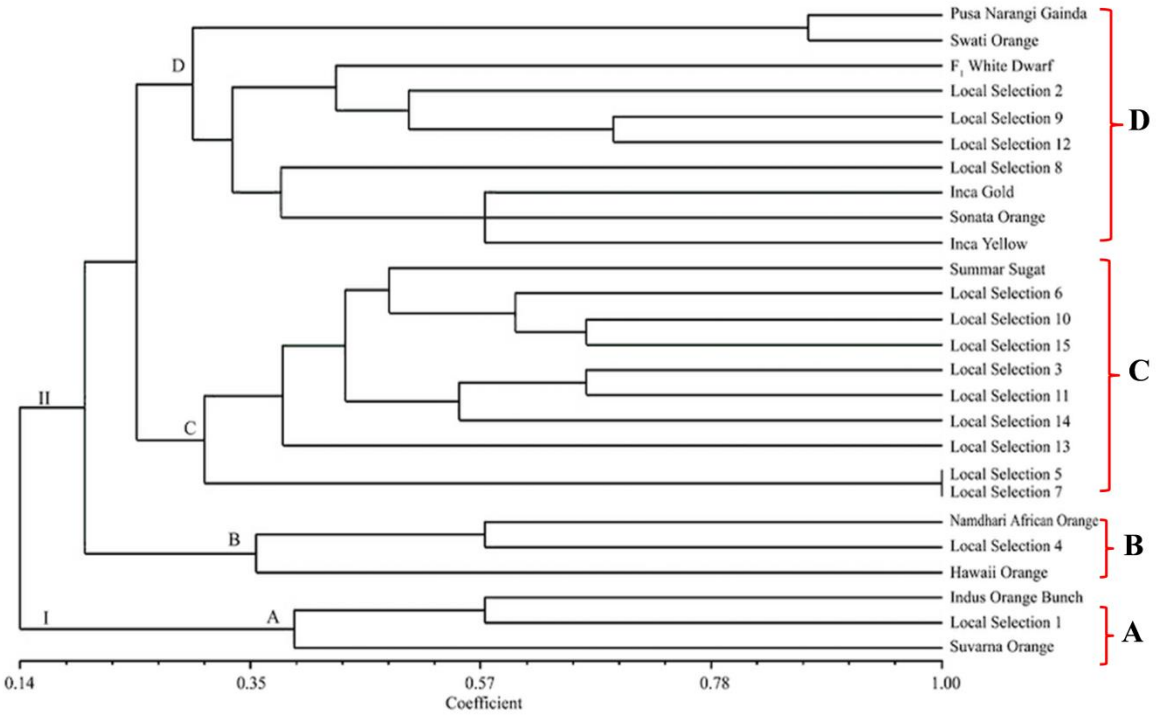

Fig. 3: Dendrogram depicting the genetic relationship among different accessions of Marigold based on RAPD data

Discussion

The present investigation on diversity analysis among marigold genotypes revealed diversity up to 34.34 per cent and around 10 primers depicted nearby 38-50 per cent polymorphism indicating vast genetic variations among the genotypes. Similar observations were made in rose[1,8,14]. Further, Scott et al[27] studied genetic variations in chrysanthemum using RAPD technology and observed that the variation between cultivars was high. These cultivars could be distinguished from each other by using different sets and the genetic variability of cultivars within and between species can also be evaluated. A wide variation was noticed among the genotypes studied in the present investigation with respect to morphological characters such as plant habit, leaf and floral characters. Renou et al[22] reported varietal identification through similar kind of study in *Pelargonium* spp., *Gerbera Jamesonii* Bolus[19] and *Turfgrass* spp.[1]

The polymorphism observed here was comparatively higher than previously observed in related species like 7% in *Tagetes minuta*[5] while lower than 70 % in *Tagetes patula*[5], 75.64% in chrysanthemum[13], 84.40 % in bougainvillea[4], 57.92 % in marigold[15], 69.51 % in sunflower[9], 89.7 % in *Chrysanthemum morifolium*[32], 68.03 in *Zinnia elegans*[34], 93.78 % in gladiolus[18], 83.33% in *Chrysanthemum grandiflorum*[12], 98.80 % in *Tagetes erecta*[25] and 100 % in Turfgrass 1. The obtained amplicons per primer for marigold are 10.21 which were almost nearer to 10.69 in marigold[15], 9.0 in gladiolus[18], 9.41 in chrysanthemum[12], 9.75 in rose[26] and 9.27 in *Tagetes erecta*[25]. The amplification profiles showing DNA banding pattern with RAPD markers are shown in fig. 2.

The data generated through RAPD markers were subjected to cluster analysis to understand their diversity and relatedness. Current study revealed two major clusters. The largest cluster including 23 genotypes was denoted as cluster-II and the smaller with 3 genotypes denoted as cluster I-A. Further, the cluster- II was found to be grouped into three sub-clusters, cluster II-B, II-C and II-D. The cluster I-A consisted of 3 genotypes namely Indus Orange Bunch, local selection 1 and Suvarna Orange. These findings are parallel to the study distinguishing all the rose accessions and species grouping them into their respective sections. Likewise, Wolff and Peters[31] detected high variation between cultivars using RAPD primers in chrysanthemum and formed three major clusters.

Cluster II is divided into three sub-clusters, II-B consisted of Namdhari Orange, Local Selection 4 and Hawaii Orange; cluster II-C consisted of varieties named Summer Sugat, Local Selection 6, Local Selection 10, Local Selection 15, Local Selection 3, Local Selection 11, Local Selection 14, Local Selection 13, Local Selection 5 and Local Selection 7 and cluster II-D involved Pusa Narangi Gainda, Swati Orange, F1 White Dwarf, local selection 2, local selection 9, local selection 12, local selection 8, Inca Gold, Sonata Orange and Inca Yellow. The phylogenetic tree revealed major diversity amongst different varieties.

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. The current findings are in accordance

with the findings of Namita et al[16], Prasad et al[20], Kaur et al[10], Kumar et al[13] and Sapna et al[25] in marigold.

Conclusion

The diversity study of germplasm is a prerequisite for crop improvement and systematic study. The present significant diversity has been reported among 26 accessions of marigold through RAPD markers which imply that these molecular markers are effective tools to discriminate various marigold genotypes. Moreover, analyses of more number of primers are recommended to evaluate the present set of accessions.

Screening of more marigold accessions with RAPD primers may give further divergence to develop SCAR markers and to identify QTL for the essential oils in marigold which would be useful for commercial exploitation. Further more specific molecular markers should be tested viz. ISSR and SSR.

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