IJPS INTERNATIONAL JOURNAL OF PLANT SCIENCES
ISSN-0973-1547 Volume **14** | Issue 1 | January, 2019 | 44-50
DOI: 10.15740/HAS/IJPS/14.1/44-50
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RESEARCH ARTICLE

RAPD assisted diversity analysis of scented geranium lines (DNA finger printing)

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SUMMARY

Scented geranium (*Pelargonium* spp.: Geraniaceae) is an important, high value aromatic crop of South African origin. Due to high demand and price for the oil, an excellent potential exists for increasing cultivated area in India. An attempt was made here to evaluate and characterize the available accessions of scented geranium based on their molecular (genetic) diversity. The present study was done using 14 accessions of scented geranium for RAPD analysis. Twenty arbitrary 10-mer oligonucleotide primers amplified 220 markerks, out of which 119 were polymorphic. Each primer amplified on an average five polymorphic bands. The total number of bands produced by each primer ranged from 7 to 16 with an average of 11 bands per primeramong which, The primers OPF-2, OPF-4, OPG-11, OPG-18, OPX-9, OPX-11, OPX-13, OPX-14 and OPX-15 proved much more useful in differentiating the accessions. The different accessions used in the investigation were grounded into two major clusters. Group I comprises of 13 accessions where it has been further subdivided into two subgroups. Subgroup I comprises of PG-1, PG-7, Kolar-C, CIMAP, Hemanti, Kunti, Kodaikanal Bourbon, PG-12, Bipuli and Kolar-N. Among these, PG-12 and Bipuli are closely related at a linkage distance of approximately 15, Kolar-N is related to these two, whereas PG-1 and PG-7 are related by a linkage distance of approximately 24, which are linked to Kolar-C and CIMAP. Kunti and Kodiakanal Bourbon formed a cluster and are linked by a distance of approximately 43. Subgroup II of Group I consists of PG-8, PG-10 and PG-11. Among these, PG-8 and PG-10 originated from a single node with a linkage distance of approximately 37, whereas, PG-11 is related to these two. Kelkar, which stood apart in a separate node in comparison with the remaining 13 acccessions. The linkage distance among the accessions as revealed by distance matrix ranged from 15 to 164. The cluster analysis (Dendrogram) using ward's method also revealed that, PG-12 and Bipuli are more related to each other and KN appears to be related to these two. PG-1 and PG-7, which were closely related, showed similarly in leaf morphology and growth pattern. Kolar-C and CIMAP are linked to these two. Hemanti' was linked with PG-1 PG-7, KC and CIMAP. The principal component analysis (PCA) also clearly shows the distinctiveness of the accession Egyptian (Kelkar) from the rest, while the grouping of different accessions is similar to the one, which is obtained in dendrogram.

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Key Words : Geranium, Accessions, Genetic diversity, RAPD analysis

How to cite this article : Harish, Singh, V.P., Ravishankar, K.V., Divyashree, N. and Yashaswini, N.P. (2019). RAPD assisted diversity analysis of scented geranium lines (DNA finger printing). *Internat. J. Plant Sci.,* **14** (1): 44-50, **DOI: 10.15740/HAS/IJPS/14.1/44-50,**

Article chronicle : Received : 24.10.2018; **Revised :** 11.12.2018; **Accepted :** 21.12.2018

Scented geranium (*Pelargonium* spp.: Geraniaceae) is an important, high value perennial aromatic crop of South African origin, that was introduced into India during 1900-1915 AD supposedly by French planters and got acclimatized to South Indian climate.

India biodiversity coupled with its vast resources including competitive workforce, highly intelligent scientific and rich business community make our country the best choice for growing aromatic crops for world market. The field or aromatic crops is assuming importance because of growing demand for natural flavours throughout the world. Many exotic and indigenous essential oils are being utilized by fragrances are industries to create high grade perfumes.

The oil has a fine rosy odour and a rich long lasting sweet rosy dry out note (Sastry *et al.*, 2000). More than 120 constituents were identified in the oil, the major ones being citronellol, geraniol, linalool, isomenthone, citronellyl formate, citronellyl acetate, geranul formate, geranyl acetate, guia 6, 9-diene, 10-epi-γ- eudesmol etc. due to high demand and price for the oil, an excellent potential exists for increasing cultivated area in India (Rao, 2000).

Presently, the classification of geranium is based on morphology and/or chemical composition of the oil. The morphological characteristics and physio-chemical properties of the oil are key issues in characterizing the genotypes/accessions, but most of the times these characters are highly influenced by environmental conditions. Hence, the data obtained by such evolutions are not easily understood at generic level, often resulting in maintenance of duplicate accessions/genotypes. So, characterizing the generic diversity existing in various genotypes/ accessions of a species is of great importance especially to plant breeders.

Molecular markers offer a unique advantage to study the diversity at DNA level. Among the various DNA markers available, RAPD (Randomly Amplified Polymorphic DNA) markers are found to be more suitable for diversity analysis since they are faserm cheaper, reproducible and able to detect even minute variations at molecular level. An attempt was made here to evaluate and characterize the available accessions of scented geranium based on their molecular diversity.

MATERIAL AND METHODS

The molecular diversity as revealed by randomly amplified polymorphic DNA (RAPDs) was carried out at the Division of Biotechnology, Indian Institute of Horticultural Research, Hessaragatta lake, Bangalore. This experiment was conducted using fourteen geranium accessions (Table A).

DNA is very important for RAPD analysis, so in order to isolate DNA, first the sample preparation was done where, The young healty leaves of geranium were collected from the potted plants in brown paper cover and washed thoroughly with double distilled water and air-dried to remove moisture. 1.0 g of leaf tissue was taken for DNA extraction.

DNA was extracted following CTAB [Cetyl trimethy] ammonium bromide] method the protocol is

Table A : Geranium accessions used for RAPD analysis

Sr. No.	Accession	Maintained at	Original sources
1.	PG-1	IIHR, Bangalore	Govt Cinchona Dept., Ooty
2.	PG-7	IIHR, Bangalore	HRS Kodaikanal
3.	PG-8	IIHR, Bangalore	*
4.	PG-10	IIHR, Bangalore	*
5.	PG-11	IIHR, Bangalore	Farmer field Ooty
6.	PG-12	IIHR, Bangalore	*
7.	CIMAP	GKVK	CIMAP
8.	KodaikanalBourrbon	IIHR	CIMAP
9.	Kelkar [Egyptian]	Hyderabad	Egypt (CIMAP, Hyderabad)
10.	Hemanti	CIMAP Kodaikanal	CIMAP Kodaikanal
11.	Kunthi	CIMAP Kodaikanal	*
12.	Bipuli	CIMAP Kodaikanal	*
13.	Kolar – C	IIHR, Bangalore	Farmer's field Kolar
14.	Kolar -N	IIHR, Bangalore	Farmer's field Kolar

given below.

– 10 ml extraction buffer was preheated to 60°C for 15-20 min (with 0.2% β – Mercaptoethanol)

– Leaf tissue [1 g] [polyvinyl pyrollidine powder] and mixed. Transferred the contents into centrifuge tubes [15ml] containing 10ml CTAB [Cetyltrimethyl ammonium bromide] buffer purchased to 60°C later the tubes were inverted several times gently.

– The tubes were included for 1 hr at 60°C with intermittent shaking, cooled to room temperature.

– 10ml of chloroform: isoamyl alcohol [24:1] were added .

– The contents were mixed gently by inverting the tubes about 25 times to form an emulsion.

– Spun at 5000 rpm for 15 minutes and the aqueous phase was transferred to new centrifuge tubes using a wide bore pipette tip.

– The clear aqueous phase was transferred to fresh centrifuge tubes.

– 2.5ml [0.5 volume] of 5M NaCl was added to the aqueous solution recovered from the steps and mixed well.

– The two volumes of 95 percent cold ethanol was and refrigerated at - 20°C for 1 to 2 hrs or until DNA stands begins to appear.

– The tubes were centrifuged at 3000 rpm for 3 min and then at 5000 rpm for 3 min at room temperature.

– The supernatant was discarded and the DNA pellet washed with cold ethanol [76%], Ethanol was removed completely without allowing the DNA pellet to dry completely by leaving uncovered at 37°C for 20 to 30 min.

– The DNA pellet was dissolved in 200 u 1 TE [TRIS-EDTA] buffer.

– This was then treated with RNase at a final concentration of 10 µg/ml [2 ul concentrated RNase/ml] and incubated at 37°C for 30 min.

– Then the sample was diluted with 1 ml TE buffer. Added 1 ml of 7.5 M ammonium acetate followed by 5ml cold ethanol [95-100%] and then mixed gently to precipitate DNA. Keep it overnight or 1 hr at-20°C.

– Centrifuge at 12000 rpm for 20 min in a refrigerated centrifuge at 4°C.

– Drainout the supernatant, air dry sample at 37°C for 15 min, suspend in 300 µl TE buffer and transfer the DNA solution into 1.5 ml microfuge tubes and store at -20°C.

– Physical integrity of DNA was verified by electrophoresis on 0.8 per cent agarose gel (60V for three hours). Finally, the DNA was quantified by using UV spectrophotometer.

DNA recovery varied widely ranging from 30 to 183 µg / g of leaf tissue. The ratio of DNA proteins ranged from 1.51 to 2.5 (Table D).

DNA amplification by polymerase chain reaction (PCR):

The PCR procedure as described by Williams *et al.*, 1990 was followed with minor modifications. A single decamer primer of arbitrary sequence was used for RAPD analysis. Amplification was carried out in 25 µl reaction mixture containing template DNA(50 ng), primer (0.3 µM), $MgCl_2$ (2.2mM), *Taq* DNA polymerase (0.5 units) with 10 x buffer and DNTPs amplification. Later twenty primers were used for RAPD analysis as listed in Table B. The amplification conditions are listed in Table C.

For RAPD analysis, concentration of 50 ng temple DNA, 2.2 mM $MgCl_2$, 100 µM dNTPs and 0.3 µM primer were found optimum, by which quality amplification and reproducible banding patterns could be obtained.

Amplification conditions for each cycle of PCR consisted of three steps, which were repeated 35 times

Table B: Random primers used in RAPD analysis and their sequence

Sr. No.	Primer code	Sequence 5' to 3'
1.	OPF 02	GAGGATCCCT
2.	OPF 04	GGTGATCAGG
3.	OPF 05	CCGAATTCCC
4.	OPG 08	TCACGTCCAC
5.	OPG 16	AGCGTCCTCC
6.	OPG 17	ACGACCGACA
7.	OPG 18	GGCTCATGTG
8.	OPG 19	TGCCCGTCGT
9.	OPV 01	GTGGCATCTC
10.	OPV 04	GGCTGCAATG
11.	OPV 11	AGACGATGGG
12.	OPX 07	GAGGGAGGCT
13.	OPX 08	CAGGGGTGGA
14.	OPX 09	GGTCTGGTTG
15.	OPX 11	GGAGCCTCAG
16.	OPX 12	TCGCCAGCCA
17.	OPX 13	ACAGGTGCAA
18.	OPX 14	ACAGGTGCTG
19.	OPX 15	CAGACAAGCC
20.	OPX 17	GACACGGACC

Table C : Amplification conditions for RAPD

Sr. No.	Condition	Temperature	Duration
1.	Initial denaturation	93°C	2 min
2.	Denaturation	92°C	2 sec
3.	Annealing	35°C	2 sec
4.	Polymerization	72°C	1.5 min
5.	Repeat step 2 to 4	35 Times	--
6.	Extended polymerization	72°C	8 min
7.	Refrigeration	4°C	

Table D : DNA content of the samples and relative ratio of DNA to proteins

Sr. No.	Geranium accessions	260 nm A_1	280 nm A_2	A_1 / A_2	DNA content (µg/g of leaf tissue
1.	PG-1	0.090	0.056	1.6071	90
2.	PG-7	0.070	0.039	1.7948	70
3.	PG-8	0.138	0.090	1.5333	138
4.	PG-10	0.084	0.051	1.6470	84
5.	PG-11	0.153	0.100	1.5300	153
6.	PG-12	0.065	0.043	1.5116	65
7.	Bipuli	0.022	0.013	1.6923	44
8.	Hemanti	0.145	0.102	1.5196	145
9.	Kolar – N	0.015	0.006	2.5000	30
10.	Kolar – C	0.154	0.100	1.5400	154
11.	CIMAP	0.140	0.090	1.5555	140
12.	Kelkar	0.139	0.091	1.5274	139
13.	Kunti	0.183	0.101	1.8087	183
14.	Kodaikenal bourbon	0.140	0.091	1.5384	140

For quantifying DNA, 10µl was taken in 990 µl of TE buffer and then absorbency was recorded using a UV Spectrophotometer

for RAPD analysis.

Denaturation, Annealation and Extenstion.

Estimation of genetic relatedness of different geranium accessions using RAPD markers:

Amplified RAPD fragments were separated on 1.5 per cent agarose gel containing ethidium bromide (0.5 µg/ml). The reaction volume of 25 µl along with 2.5 µl of sample buffer was loaded into the wells. Electrophoresis was conducted at 60V (5V/cm) for 3 to $3_{1/2}$ hrs. The agarose gels were viewed under UV light and were photographed in a gel documentation system.

Data analysis:

The RAPD bands were scored for its presence as '1' and absence as '0' at each position. The data were analyzed for dendrogram, distance matrix and Principal component analysis (PCA), based on minimum variance algorithm (Ward, 1963) and squared euclidean distances (Sokal and Sneath, 1963).

RESULTS AND DISCUSSION

In the present study fourteen scented geranium accessions were grouped using data from twenty arbitrary 10-mer oligonucleotides primers. RAPD profiles were generated for 34 genotypes of *Pelargonium* with 23 primers (Renou *et al.*, 1997) that allowed all genotypes to be distinguished from each other. Twenty arbitrary 10-mer oligonucleotide primers amplified 220 markerks, out of which 119 were polymorphic. Each primer amplified on an average five polymorphic bands. The total number of bands produced by each primer ranged from 7 to 16 with an average of 11 bands per primer. The details regarding the primers used for amplification and the bands announced by them are presented in Table 1. The primers OPF2, OPF4, OPG-

11, OPG-18, OPX-9, OPX-11, OPX-13, OPX-14 and OPX-15 proved much more useful in differentiating the accessions. Linkage among the 14 accessions revealed by cluster analysis using Ward's method.

During the study, 60 random 10 base long primers (Operon Technologies, USA) were screened using pooled geranium DNA, which on an average gave 4 bands per primer. The primers, which gave multiple bands, as listed in Table 1 were selected for diversity studies.

The different accessions used in the investigation were grounded into two major clusters. Group I comprises of 13 accessions. This group has been further subdivided into two subgroups. Subgroup I comprises of PG-1, PG-7, Kolar-C, CIMAP, Hemanti, Kunti, Kodaikanal Bourbon, PG-12, Bipuli and Kolar-N. Among these, PG-12 and Bipuli are closely related at a linkage distance of approximately 15, Kolar-N is related to these two, whereas PG-1 and PG-7 are related by a linkage distance of approximately 24, which are linked to Kolar-C and CIMAP. Kunti and Kodiakanal Bourbon formed a cluster and are linked by a distance of approximately 43. Subgroup II of Group I consists of PG-8, PG-10 and PG-11. Among these, PG-8 and PG-10 originated from a single node with a linkage distance of approximately 37, whereas, PG-11 is related to these two.

Group-II comprises of only one accession *i.e.*, Kelkar, which stood apart in a separate node in comparison with the remaining 13 accessions. The linkage distance among the accessions as revealed by distance matrix (Table 2) ranged from 15 to 164.

Principal component analysis also revealed that, all the accessions except Kelkar, are grouped together. The variation in the PCA of 14 geranium accessions was 63.6 per cent (Eigen value).

The cluster analysis (Dendrogram) grouped the accessions into two major groups, under group I , PG-12 and Bipuli are more related to each other and KN appears to be related to these two. This could be due to the similarity in their chemical composition especially the major constituents such as citronellol, geraniol, linalool etc. the cultivar Bipuli possessed 26.3 per cent citronellol, 33.2 per cent geraniol and 6 to 8.6 per cent linalool as reported by Kaul and Rao (1999), whereas the concentration of these three components are 21.245 (Citronellol), 30.205 per cent (geraniol) and 8.703 per cent (linalool) in the accession PG-12 .

PG-1 and PG-7, which were closely related, showed similarly in leaf morphology and growth pattern. Kolar-C and CIMAP are linked to these two as shown in

Table 1: Nature and number of bands produced by different primers

Sr. No.	Primer code	Number of monomorphic bands	Number of polymorphic bands	Total number of bands
1.	OPF 02	7	9	16
2.	OPF 04	5	7	12
3.	OPF 05	6	5	11
4.	OPG 11	2	7	9
5.	OPG 16	6	4	10
6.	OPG 17	4	7	11
7.	OPG 18	5	7	12
8.	OPG 19	2	7	9
9.	OPV 01	2	5	7
10.	OPV 04	7	2	9
11.	OPV 11	5	5	10
12.	OPX 07	5	5	10
13.	OPX 08	3	4	7
14.	OPX 09	2	9	11
15.	OPX 11	7	7	14
16.	OPX 12	6	5	11
17.	OPX 13	4	7	11
18.	OPX 14	6	7	13
19.	OPX 15	7	7	14
20.	OPX 17	9	3	12

dendrogram, which is due to the similarly in their morphology or might be due to the chemical composition of the essential oil.

'Hemanti' was linked with PG-1, PG-7, KC and CIMAP. The cultivar 'Hemanti' is an Algerian type rich in citronellol (Sastry *et al.*, 2000). The accession PG-1 is also an Algerian type and also found to be rich in citronellol (Bhaskar *et al.*, 1998). This might be the reason for the close association of Hemanti with PG-1 and other accessions *i.e.*, PG-7, KC and CIMAP.

PG-8, PG-10 and PG-11 formed a separate subgroup in the group I. Among these PG-8 and PG-10 were very close, while PG-11 is related to these two. It was observed in the present study that PG-8 and PG-10 contained relatively equal amounts of citronellyl formate (15.129 and 15.236%, respectively) and citronellol (25.2 and 29.5% in PG-8 and PG-10) .

The accession PG-11 which is related to PG-8 and PG-10 also possessed 13.818 per cent of citronellyl formate and 21.874 per cent citronellol (Table 3). Closeness of these accessions may be also due to the fact that they might have been originated from a single parent.

Group II consists of only one accession *i.e.*, Egyptian (Kelkar), which was basically introduced to India from Egypt. The oil of Egyptian (Kelkar) type contains geraniol in about 2 to 5 fold more of citronellol (Sastry *et al.*, 2000) and in case of other accessions, the geraniol concentration is about 1 or 2 fold axcess and / or lower as observed in the present study. It was clear from the

Table 2 : Distance matrix analysis of scented geranium accessions

Sr. No.	Geranium accessions	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	PG-1	0.00	24.00	47.00	54.00	51.00	56.00	61.00	52.00	65.00	58.00	42.00	150.00	55.00	60.00
2.	PG-7	24.00	0.00	49.00	60.00	65.00	40.00	43.00	48.00	45.00	40.00	34.00	148.00	47.00	52.00
3.	PG-8	47.00	49.00	0.00	37.00	44.00	73.00	74.00	67.00	76.00	67.00	63.00	155.00	54.00	65.00
4.	PG-10	54.00	60.00	37.00	0.00	43.00	82.00	81.00	58.00	85.00	60..	68.00	158.00	63.00	66.00
5.	PG-11	51.00	65.00	44.00	43.00	0.00	87.00	92.00	61.00	86.00	77.00	75.00	159.00	62.00	69.00
6.	PG-12	56.00	40.00	73.00	82.00	87.00	0.00	15.00	58.00	27.00	48.00	36.00	158.00	55.00	52.00
7.	Bipuli	61.00	43.00	74.00	81.00	92.00	15.00	0.00	57.00	26.00	49.00	41.00	163.00	58.00	55.00
8.	Hemanti	52.00	48.00	67.00	58.00	61.00	58.00	57.00	0.00	65.00	50.00	56.00	164.00	61.00	66.00
9.	Kolar – N	65.00	45.00	76.00	85.00	86.00	27.00	26.00	65.00	0.00	59.00	41.00	159.00	60.00	63.00
10.	Kolar – C	58.00	40.00	67.00	60.00	77.00	48.00	49.00	50.00	59.00	0.00	40.00	154.00	57.00	60.00
11.	CIMAP	42.00	34.00	63.00	68.00	75.00	36.00	41.00	56.00	41.00	40.00	0.00	138.00	43.00	52.00
12.	Kelkar	150.00	148.00	155.00	158.00	159.00	158.00	163.00	164.00	159.00	154.00	138.00	0.00	125.00	148.00
13.	Kunti	55.00	47.00	54.00	63.00	62.00	55.00	58.00	61.00	60.00	57.00	43.00	125.00	0.00	43.00
14.	Kodaikenal Bourbon	60.00	52.00	65.00	66.00	69.00	52.00	55.00	66.00	63.00	60.00	52.00	148.00	43.00	0.00

Table 3: Principal components (%) of the steam distilled oils of scented geranium accessions

Sr.	Accessions	PG-1		PG-8		PG-10		PG-11		PG-12		KB		CIMAP	
No.	Components	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1.	Isomenthone	7.717	5.708	7.240	5.324	8.500	7.739	6.560	5.081	7.398	4.724	6.163	5.802	6.439	5.913
2.	Linalool	8.644	7.371	8.614	6.546	3.675	2.413	9.787	8.349	8.583	8.703	6.952	7.863	7.573	7.003
3.	Citronellyl formate	15.534	11.903	15.129	12.377	15.236	23.183	13.818	10.716	12.104	11.516	11.222	13.583	7.276	12.290
4.	Citronellyl acetate	13.831	12.251	12.248	13.075	3.361	4.925	14.073	13.154	10.751	13.19	10.315	12.348	4.059	12.290
5.	Citronellol	18.522	22.314	17.352	21.341	23.529	37.073	21.874	17.674	18.467	21.246	31.026	23.061	26.859	22.662
6	Geraniol	15.743	19.957	14.536	19.461	5.095	4.362	13.748	20.505	15.495	17.587	20.559	20.393	26.285	19.662
7.	Neryl formate	0.045	0.096	0.117	0.366	3.361	0.751	0.106	0.365	0.231	0.299	0.079	0.287	T	T
8.	Geranyl formate	T	0.955	0.729	0.189	4.908	0.726	T	0.475	3.691	0.717	0.220	0.886	0.936	0.361
9.	Nerol	0.877	0.261	0.261	0.390	4.733	2.651	2.361	0.263	1.838	0.253	0.440	0.145	0.563	0.142
10.	Geranyl acetate	0.944	0.128	0.730	0.935	2.362	1.373	0.905	0.692	0.865	0.452	T	0.496	2.353	1.096

A: The oil distilled during May 2001 B: The oil distilled during Aug. 2001, T: Traces

dendrogram that Egyptian (Kelakar) was very distinct from the rest of the accessions, which might be to due to the fact that it was introduced to India very recently, whereas, the other accessions have been introduced long back and acclimatized well and was very rich in geraniol which is not so in case of other accessions.

It was interesting to note that some of the accessions (PG-11 and PG-12; PG-7 and PG-10 and PG-1 and PG-8) were similar in morphology but differed in their chemical composition of the oil and odour characteristics. Still these accessions do not from close association in the dendrogram. Further, it is not always necessary that the accessions possessing similar morphology should be closely related. Similar results were obtained by Padmesh *et al.* (1999) in *Andrographis paniculata.*

The principal component analysis (PCA) clearly shows the distinctiveness of the accession Egyptian (Kelkar) from the rest, while the grouping of different accessions is similar to the one, which is obtained in dendrogram.

Conclusion:

Genetic relatedness as revealed by RAPD markersare:

– Dendrograms based on the banding patterns of different accessions revealed that there was a considerable degree of genetic diversity exists among the accessions.

– Both PCA and dendrogram analysis revealed the distinctive nature of the accession Egyptian (Kelkar) which was recently introduced to India from Egypt by M/s Kelkars, a prominent perfumery house in India.

– It was very clear from this study that the accessions, which were similar in morphology, may not be similar genetically.

– The utility of RAPD markers in assessment of genetic relatedness in scented geranium has been successfully demonstrated.

– All the accessions under the present study though have some mporphological resemblance to each other are genetically distinct and unique.

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