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Molecular diversity study within holy basil species (*Ocimum tenuiflorum* L.) using ISSR and RAPD markers

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ABSTRACT

In the present study, molecular diversity among the 38 accessions of holy basil (*Ocimum tenuiflorum*) collected from four phyto-geographical regions of India was assessed using RAPD and ISSR markers. A total of 80 fragments were amplified with 19 selected RAPD primers (4.21 fragments amplified per primer), of which 66 (82.50%) were polymorphic. The resolving power (Rp) for RAPD ranged from 2.32 to 8.96 with an average of 5.48 per primer. Similarly, nine ISSR primers amplified a total of 25 scorable fragments (2.77 fragments amplified per primer) out of which 18 fragments amplified (72.00%) were polymorphic. The Rp for ISSRs ranged from 2.4 (UBC-807) to 9.95 (UBC-886). For genetic diversity and genetic relationship study, the fragments amplified by both the marker systems were pooled for analysis. Jaccard's similarity coefficient was used to estimate the genetic diversity and it varied from 0.0259 to 0.935. Cluster analysis was done using UPGMA method which grouped all 38 accessions into four clusters. Genetic relationship based on RAPD and ISSR markers show that all 38 accessions do not show any genetic isolation based on phyto-geographical regions of India. Analysis of molecular variance (AMOVA) study shows that 9% of variation was present between the phyto-geographic regions whereas, the maximum variation was observed between the accessions (91%). Principal Coordinate Analysis (PCoA) showed that large diversity existed in *O. tenuiflorum* collection and percentage of variation explained by the first 3 axes was 60.57%. The diversity present within *O. tenuiflorum* species can be exploited for breeding and diversity conservation.

Key words: Genetic diversity, ISSR, *Ocimum tenuiflorum*, RAPD.

INTRODUCTION

Genus *Ocimum* (Lamiaceae; formerly Labiatae) or Holy basil, *Tulsi* botanically called *O. tenuiflorum* L. (syn. *O. sanctum* L.; chr. No. 2n = 32) is a very important species for Indian region. *O. tenuiflorum* is native of India and widespread as a cultivated plant and an escaped weed, covering entire Indian sub-continent ascending up to 1,800 m in the Himalaya and the Andaman and Nicobar islands in the south (Kirtikar and Basu, 4). The two main morphotypes of *O. tenuiflorum* cultivated in India and adjoining regions are light green-leaved (Sri Rama or Lakshmi Tulsi) is bigger in size and purple-leaved (Sri Krishna Tulsi) shorter in size or dark green-magenta leaves (Kothari *et al*., 5). Holy accessions from northern India have reported high percentage of eugenol variation in percentage of active chemical compounds in *O. tenuiflorum* (Raina *et al*., 11).

Characterization of accessions provides description of the material essential for their identification, conservation, management and utilization in crop improvement programs. Chen *et al*. (2) carried out, genetic diversity analysis of 37 basil accessions, which belongs to four *Ocimum* species using three marker systems, i.e. RAPD, ISSR and SRAP. This study has demonstrated that ISSR, RAPD and SRAP individually and in combination were efficient tools to study genetic diversity in basil accessions. Singh *et al*. (14) carried out study on five *Ocimum* species for phylogenetic relationships using RAPD markers. This study revealed high degree of polymorphism (98.28%) among the 32 accessions. Vieira *et al.* (15) investigated the genetic relationship between seven species of basil using RAPD primers and found close relation between *O. basilicum* and *O. tenuiflorum*. Six different species of *Ocimum* were studied by Lal *et al*. (6) using RAPD, ISSR and SSR markers. The literature survey shows that maximum study has been undertaken to study the genetic diversity present between the *Ocimum* sp. using different molecular markers. At present, only few studies were recorded on the diversity analysis within *O. tenuiflorum* species using DNA markers. Hence, the present study has been undertaken to assess genetic diversity present in *O. tenuiflorum* species.

MATERIALS AND METHODS

The experimental material consisted of 38 accessions of *O. tenuiflorum* representing four

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phyto-geographical regions (Arora, 1) belonging to eight states of India (Table 1). The material was grown in the experimental pots in the net-house at NBPGR, New Delhi during June-September, 2013. For molecular characterization, the leaf samples were collected from young plants and stored at -80°C. DNA was extracted from the leaf tissues using CTAB (Cetyl trimethyl ammonium bromide) method of Saghai-Maroof *et al*. (13) with modification. Leaves were bulked from five plants of same accessions and three grams of young and healthy leaf material per accession was frozen in liquid nitrogen (-196°C) and then crushed into fine powder using mortar and pestle. Polyvinyl pyrrolidone (PVP) was added to remove polyphenols. All samples were treated with RNase (10 mg/ ml) at a concentration of 40 ng/ml of DNA and kept for incubation at 37°C for one hour. The quality of DNA was checked on 0.8% agarose gel electrophoresis at 100 V for one hour in TAE buffer. The DNA concentration was estimated using a Nanodrop® (Thermofisher Scientific, USA) spectrophotometer. DNA samples were diluted with appropriate amount of sterilized distilled water to make a working concentration of 25 ng/µl. The diluted samples were stored at 4°C for immediate use.

Initially, 100 RAPD primers were screened for polymorphism and primers which produced consistent result were used for the generation of profiles of all the 38 accessions. Finally, 19 RAPD primers were used for final profile generation. PCR amplification was optimized and reaction mixture of 25 µl containing 1x PCR buffer, 2.5 mM of $MgCl_2$, 0.2 mM of dNTPs (dATP, dCTP, dTTP and dGTP), 1U *Taq* polymerase (Fermentas, USA) and 30 ng DNA was used. Amplification was carried out in a thermo-cycler (G-STORM, UK) with thermal cycle condition for RAPD markers as follows: Initial denaturation at 94°C for 5 min. was followed by 42 cycles of denaturation at 94°C for 1 min., primer annealing at 37°C for 2 min., extension for 2 min. at 72°C and final extension at 72°C for 7 min. After the amplification products were cooled at 4°C, they were separated on a 1.4 per cent agarose gel prepared in 1x TAE buffer. GeneRuler™ 1 Kb ladder (0.1 µg/µl) were used as standard. The fragments amplified were visualized and documented using gel documentation system (Syngene, UK).

Forty seven ISSR primers were screened of which, 9 ISSR primers were used for final profile generation. Reaction mixture of 25 µl containing ingredients same as mentioned above for RAPD was used. Amplification was carried out in a thermo-cycler with condition same as for RAPD except primer annealing at 55°C for 1 min. The amplified products were separated by agarose gel electrophoresis (1.4%) and gel was stained with ethidium bromide. GeneRuler™ 50 bp ladder (0.5 µg/µl) were used as standard.

Amplified products were scored across the lane with respect to their molecular weight. Presence of band was scored as (1) and absence of band scored as (0). Reproducible amplified fragments of RAPD and ISSR (*i.e.* those fragments amplified present in both repetitions of sample) were scored manually. Weak fragments amplified of negligible intensity and smeared fragments amplified were excluded from the final data analysis. The ability of a primer or technique to distinguish between large numbers of genotypes, *i.e.* Resolving power (Rp) of the primer of RAPD and ISSR primers were determined as described by Prevost and Wilkinson (10). Genetic relationships among the accessions were calculated using the software program NTSYSpc2.1 (Rohlf, 12). Pairwise comparison between accessions were done using Jaccard's similarity coefficient. UPGMA based dendrogram showing genetic relationships among accession were also constructed using the same software. To measure reliability of the resulting genotypic groups, the original matrix was bootstrapped 1000 times by employing Winboot in order to group the accessions into discrete clusters. Bootstrap values obtained with Winboot were placed on the nodes of cluster on the dendrogram generated by NTSYSpc 2.1. Principal Coordinate Analysis (PCoA) and Analysis of Molecular Variance (AMOVA) were performed using software GenAlEx V6.5 (Peakall and Smouse, 9).

RESULTS AND DISCUSSION

In the present investigation, *O. tenuiflorum* accessions having different morphological and biochemical characters already reported (Malav *et al*., 7; Raina *et al*., 11) were analysed based on RAPD and ISSR markers. The 38 *O. tenuiflorum* accessions collected from four phyto-geographical regions belonging to eight states of India (Table 1). Out of 100 RAPD and 47 ISSR primers initially screened, 19 RAPD primers and 9 ISSR primers that showed a clear and reproducible band patterns were chosen for generation of markers profiles.

A total of 80 fragments were amplified with 19 selected RAPD primers. Maximum number of fragments (7 fragments) was amplified with primer M-16 whereas; the minimum number of fragments (2 fragments) was amplified with N-12 and O-3 primers (Table 2). On average 4.21 fragments per primer were amplified and out of 80 fragments, 66 (82.50%) were polymorphic with an average of 3.47 fragments amplified per primer (Table 2). The

Table 1. List of *Ocimum tenuiflorum* accessions used in the study.

S. No.	Acc. No.	District	State	Phyto-geographical region*
1	KCB-2	North Cachar Hills	Assam	Eastern region
2	KCB-4	North Cachar Hills	Assam	Eastern region
3	KCB-8	North Cachar	Assam	Eastern region
4	KCB-21	North Cachar Hills	Assam	Eastern region
5	KCB-25	North Cachar Hills	Assam	Eastern region
6	PM/12/1	Patna	Bihar	Gangetic plain
7	PM/12/4	Delhi	New Delhi	Gangetic plain
8	PM/12/5	Patna	Bihar	Gangetic plain
9	IC583278	Mathura	Uttar Pradesh	Gangetic plain
10	IC583279	Mathura	Uttar Pradesh	Gangetic plain
11	IC583280	Mathura	Uttar Pradesh	Gangetic plain
12	IC583281	Mathura	Uttar Pradesh	Gangetic plain
13	IC583282	Mathura	Uttar Pradesh	Gangetic plain
14	IC583283	Mathura	Uttar Pradesh	Gangetic plain
15	IC583284	Mathura	Uttar Pradesh	Gangetic plain
16	IC583285	Hathras	Uttar Pradesh	Gangetic plain
17	IC583286	Hathras	Uttar Pradesh	Gangetic plain
18	IC583288	Aligarh	Uttar Pradesh	Gangetic plain
19	IC583290	Bulandshahar	Uttar Pradesh	Gangetic plain
20	IC583293	Meerut	Uttar Pradesh	Gangetic plain
21	IC583300	Bijnor	Uttar Pradesh	Gangetic plain
22	IC583302	Bijnor	Uttar Pradesh	Gangetic plain
23	IC583303	Bijnor	Uttar Pradesh	Gangetic plain
24	IC583304	Bijnor	Uttar Pradesh	Gangetic plain
25	IC583312	Bareilly	Uttar Pradesh	Gangetic plain
26	IC583313	Bareilly	Uttar Pradesh	Gangetic plain
27	IC583314	Badaun	Uttar Pradesh	Gangetic plain
28	IC583318	Saharanpur	Uttar Pradesh	Gangetic plain
29	IC583319	Saharanpur	Uttar Pradesh	Gangetic plain
30	IC583320	Farrukhbad	Uttar Pradesh	Gangetic plain
31	IC583321	Farrukhbad	Uttar Pradesh	Gangetic plain
32	IC583322	Hathras	Uttar Pradesh	Gangetic plain
33	PM/12/3	Baran	Rajasthan	Indus plain
34	PM/12/6	Neemuch	Madhya Pradesh	Indus plain
35	IC381185	Chamba	Himachal Pradesh	Western Himalaya
36	IC583305	Udham Singh Nagar	Uttarakhand	Western Himalaya
37	IC583306	Udham Singh Nagar	Uttarakhand	Western Himalaya
38	IC583310	Udham Singh Nagar	Uttarakhand	Western Himalaya

*Phyto-geographical regions (Arora, 1991)

number of polymorphic fragments produced per primer ranged between 1 (N-11, N-12, O-3) to 6 (M-16). The fragments size ranged between 250-2000 bp. Out of nineteen RAPD primers used, eight showed 100% polymorphism, whereas, N-11 (25%) was least polymorphic (Table 2). Singh *et al*. (14) and Chen *et al*. (2) also reported high degree of polymorphism (98.28 and 95%), respectively among different species

Table 2. Details of RAPD primers used for characterization of *O. tenuiflorum* accessions.

Primer	TNB	NPB	P%	Rp
G-2	4	4	100	3.70
G-7	3	2	66.67	3.72
G-9	5	5	100	6.45
G-14	5	5	100	6.65
G-17	5	5	100	5.55
M-7	3	3	100	3.12
M-13	5	5	100	6.05
M-16	7	6	85.71	8.96
N-7	3	2	66.67	3.36
N-10	4	4	100	5.40
N-11	4	1	25	6.00
N-12	2	1	50	2.32
N-16	6	4	66.67	8.76
N-20	6	5	83.33	8.82
O-3	2	1	50	2.78
O-5	4	3	75	6.28
T-7	4	3	75	5.60
T-18	3	2	66.67	4.14
T-20	5	5	100	6.55
Total	80	66		104.21
Av.	4.21	3.47	79.51	5.48

TNB = Total No. of bands; NPB = No. of polymorphic bands; P% = Polymorphism percentage; Rp = Resolving power

Table 3. Details of ISSR markers used for characterization of *O. tenuiflorum* accessions.

S. No.	Primer	TNB	NPB	P%	Rp
1.	UBC-807	2	1	50	2.4
2.	UBC-808	3	2	66.67	5.16
3.	UBC-809	3	2	66.67	3.99
4.	UBC-860	1	0	0	0
5.	UBC-861	3	2	66.67	4.44
6.	UBC-868	1	0	0	0
7.	UBC-873	3	3	100	4.17
8.	UBC-874	4	4	100	5.16
9.	UBC-886	5	4	80	9.95
Total		25	18		38.87
Av.		2.77	2	58.89	4.31

TNB = Total No. of bands; NPB = No. of polymorphic bands; P% = Polymorphism percentage; Rp = Resolving power

of *Ocimum* using RAPD marker. The resolving power (Rp) of the 19 RAPD primers ranged from 2.32 (N-12) to 8.96 (M-16) with an average of 5.48 per primer (Table 2). The primers with the high Rp values were more informative as they were able to distinguish more number of *O. tenuiflorum* accessions.

Since RAPD and ISSR are dominant makers therefore, to assess the genetic diversity and genetic relationship the fragments amplified generated by both the marker system were pooled for analysis to get better diversity spectrum. Jaccard's similarity coefficient was used to estimate the genetic diversity present in the study and it varied from 0.0259 to 0.935. The maximum diversity was observed between IC583290 and IC583285, whereas; the minimum diversity was present between KCB-4 and IC-583303 & IC-583304. Cluster analysis was done using UPGMA method which grouped all 38 accessions into four clusters (Fig. 1). In cluster 1 and 3, two accessions were grouped, whereas, in the cluster 2 three samples were grouped. Cluster 4 was the largest cluster with

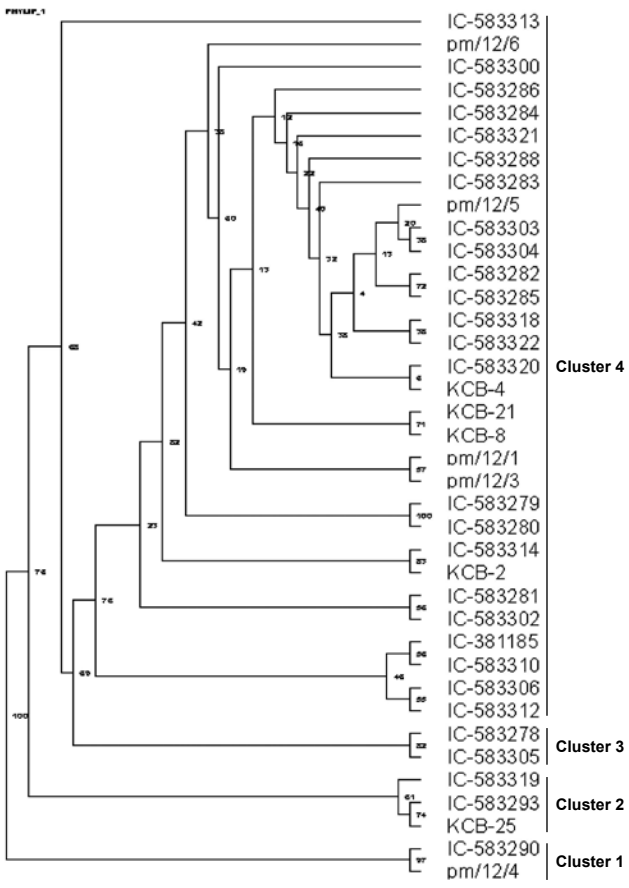

Fig. 1. Phylogenetic dendrogram of *O. tenuiflorum* accessions based on RAPD and ISSR amplification profiles.

31 accessions, which can be further subdivided into sub-clusters. Genetic relationship based on RAPD and ISSR markers shows that all 38 accessions do not show any genetic isolation which were collected from four different phyto-geographical regions of India. In cluster 2 accessions from Gangetic plain (IC583293 and IC 583319) were sharing similarity with accession collected from Eastern region (KCB-25). Similarly, in cluster 3 accession collected from Gangetic plain (IC583278) was sharing genetic similarity with accession collected from Western Himalaya (IC583305). In cluster 4 accessions from all four phyto-geographical regions were grouped together. The bootstrap value shows that cluster 1 grouping is 100% reliable and distinct from cluster 2, 3 and 4 (Fig. 1), whereas reliability for cluster 2 and cluster 3 was 76 and 69%, respectively. The bootstrap value varied 100 to 6% within the clusters. Cluster 1 had two accessions (IC583290 and PM/12/4) with 97% bootstrap value and Cluster 4 two accessions (IC583279 and IC583280) with 100% bootstrap value were found. The high bootstrap value indicated that these groupings were very reliable and will not change with addition of more markers. Chen *et al.* (2) reported four clusters for four species of *Ocimum* based on RAPD markers.

Analysis of molecular variance (AMOVA) was carried out for combined RAPD and ISSR data and 9% of variation was found among the populations (Fig. 2), which indicates less variation among four phyto-geographical regions whereas, the maximum variation was observed within populations (91%), means between the accessions. Principal Coordinate Analysis (PCoA) with combined data of RAPD and ISSR markers showed that large diversity existed in *O. tenuiflorum* collection. Accessions exhibited uniform distribution across the two axes (Fig. 3). The percentage of variation explained by the first 3 axes was 60.57%. In PCoA all accessions were labelled with

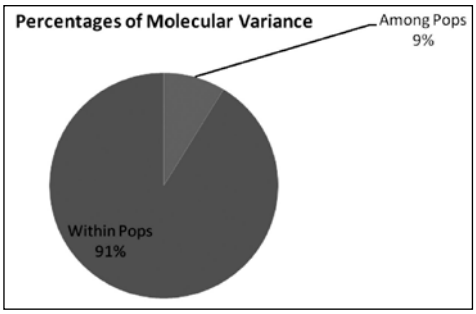

Fig. 2. Analysis of molecular variance (AMOVA) based on pooled RAPD and ISSR data.

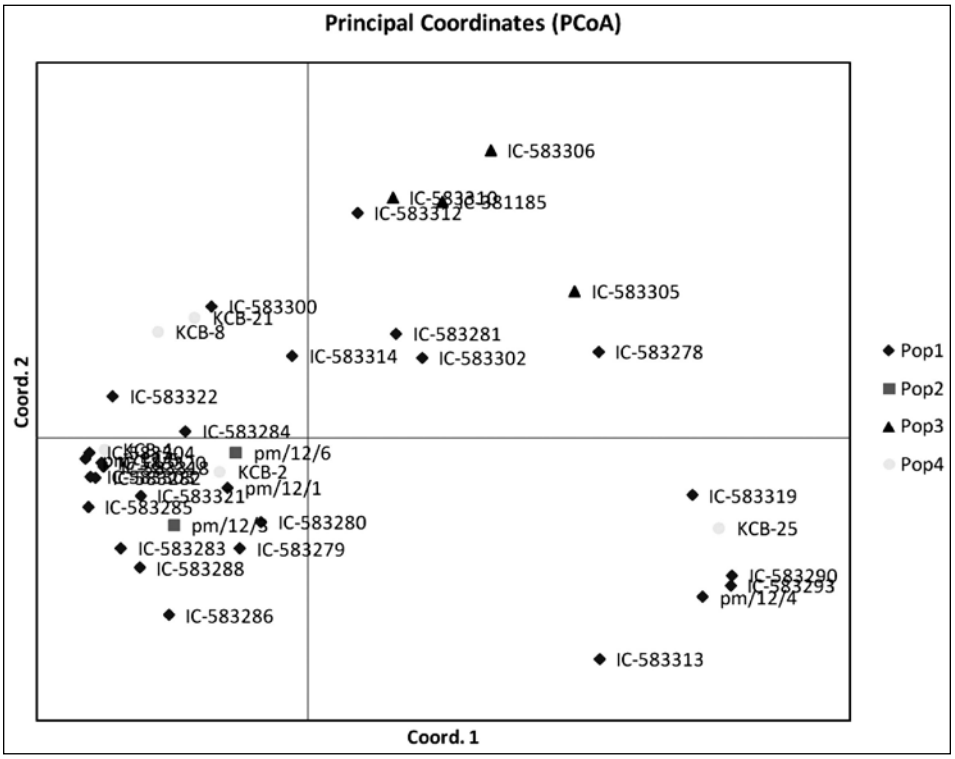

Fig. 3. Principal Coordinate Analysis (PCoA) based on pooled RAPD and ISSR data. Pop1 = Gangetic Plain, Pop2 = Indus Plain, Pop3 = Western Himalaya, Pop4 = Eastern region.

different colours based on their phyto-geographical regions to indicate their region specificity (Fig. 3), the intermixing of colour across the coordinate's, support the UPGMA tree and AMOVA analysis that, there was no location-specific grouping of the genotypes with exception of accessions collected from Western Himalaya (population 3), which were grouped together (Fig. 3).

AMOVA analysis showed that maximum variation was partitioning within population which supports UPGMA based clustering and further confirms that there is no genetic isolation based on the phyto-geographical regions and gene flow between the populations has occurred frequently. Ganesan *et al.* (3) studied *Moringa oleifera* a woody, perennial and pre dominantly out-crossed species and observed significant variation within population which was similar to our finding where maximum variation has been observed within the populations. However, there are reports, which are contradictory to above findings (Mulvi *et al*., 8). Principal Coordinate analysis based on combined RAPD and ISSR data showed that high genetic diversity was present among the 38 accessions and only samples collected from western Himalayas were grouping together. This grouping was different from the earlier grouping reported based on morphological markers by Malav *et al*. (7) based on 49 accessions of *O. tenuiflorum.* Lack of clustering according to phyto-geographical areas indicates that accessions from different geographical areas were not significantly different genetically. This may be due to spread of seed and/or high rates of gene flow between the adjacent populations.

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REFERENCES

1. Arora, R.K. 1991. Plant diversity in the Indian Gene Centre. **In**: *Plant Genetic Resources: Conservation and Management Concepts and Approaches*, Paroda, R.S. (Ed.), International Board for Plant Genetic Resources, Regional Office for South and Southeast Asia, New Delhi, India, pp. 25-54.

2. Chen, S.Y., Dai, T.X., Chang, Y.T., Wang, S.S., Ou, S.L., Chuang, W.L., Cheng, C.Y., Lin, Y.H., Lin, L.Y. and Ku, H.M. 2013. Genetic diversity among *Ocimum* species based on ISSR, RAPD and SRAP markers. *Australian J. Crop Sci.* **7**: 1463-71.

3. Ganesan, S.K., Singh, R., Choudhury, D.R., Bharadwaj, J., Gupta, V. and Singode, A. 2014. Genetic diversity and population structure study of drumstick (*Moringa oleifera* Lam.). *Indus. Crops Prod*. **60**: 316-25.

4. Kirtikar, K.R. and Basu, B.D. 1984. Indian medicinal plant. **In**: *Indian Medicinal Plants*, *Vol. 3* (2nd Edn.), Singh, B. and Singh, M.P. (Eds.), Dehradun, India, pp. 1965-68.

5. Kothari, S.K., Bhattacharya, A.K., Ramesh, S., Garg, S.N. and Khanuja, S.S. 2005. Volatile constituents in oil from different plant parts of methyl eugenol-rich *Ocimum tenuiflorum* L.f. (syn. *O. sanctum* L.) grown in south India. *J. Essen. Oil Res*. **17**: 656-58.

6. Lal, S., Mistry, K.N., Thaker, R., Shah, S.D. and Vaidya, P.B. 2012. Genetic diversity assessment in six medicinally important species of *Ocimum* from Central Gujarat (India) utilizing RAPD, ISSR and SSR markers*. I.J.A.B.R.* **2**: 279-88.

7. Malav, P., Pandey, A., Bhatt, K.C., Gopala, S. and Bisht, I.S. 2014. Morphological variability in holy basil [*Ocimum tenuiflorum* L. (Lamiaceae)] from India. *Genet. Res. Crop Evol*. DOI 10.1007/s10722-015-0227-5.

8. Muluvi, G.M., Sprent, J.I., Soranzo, N., Provan, J., Odee, D., Folkard, G., McNicol, J.W. and Powell, W. 1999. Amplified fragment length polymorphism (AFLP) analysis of genetic variation in *Moringa oleifera* Lam. *Mol. Ecol*. 8: 463-70.

9. Peakall, R. and Smouse, P. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for ting and research - an update. *Bioinformatics,* **28**: 2537-39.

10. Prevost, A. and Wilkinson, M.J. 1999. A new system of comparing PCR primers applied to ISSR finger printing of potato cultivars. *Theor. Appl. Genet*. **98**: 107-12.

11. Raina, A.P., Kumar, A. and Dutta, M. 2013. Chemical characterization of aroma compounds in essential oil isolated from ''Holy Basil'' (*Ocimum tenuiflorum* L.) grown in India. *Genet. Res. Crop Evol*. **60**: 1727-35.

12. Rohlf, F.J. 1998. NTSYS-pc: numerical taxonomy and multivariate analysis system, ver. 2.0. Applied Biostatistics Inc. New York.

13. Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A. and Allard, R.W. 1994. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. *Proc. Natl. Acad. Sci*. **81**: 8014-18.

14. Singh, A.P., Dwivedi, S., Bharti, S., Srivastava, A., Singh, V. and Khanuja, S.P.S. 2004. Phylogenetic relationships as in *Ocimum* revealed by RAPD markers. *Euphytica*, **136**: 11-20.

15. Vieira, R.F., Goldsbrough, P. and James, J.E. 2003. Genetic diversity of basil (*Ocimum* spp.) based on RAPD markers. *J. Amer. Soc. Hort. Sci.* **128**: 94-99.

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