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Genetic diversity In *Ocimumbasilicum*

K. and Ocimumtenuiflorum (L.)Keng (Lamiaceae) varieties as Revealed by RAPD analysis

Mariamma Cherian and Radhamany P. M.
Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram, Kerala, India

Abstract

Genetic diversity was studied in three populations of *Ocimumbasilicum* and nine populations of *Ocimumtenuiflorum*. Polymorphism at the molecular level was studied by Random Amplified Polymorphic DNA (RAPD) marker technique. Polymerase chain reaction with 15 decamer oligonucleotide primers was applied to the 12 test samples. All the 15 RAPD primers generated polymorphism. Six primers could generate 100% polymorphism. PCR products revealed a total of 168 bands of which 154 were polymorphic. RAPD studies revealed that the overall genetic similarity between the 12 populations is 0.67 and the similarity values ranged from 0.32 to 0.96 based on Nei and Li's similarity coefficient. Principal components analysis revealed that the first two components accounted for 59.40% of the total variation. The diversity in basils based on morphological characters as correlated with genetic diversity observed during the present study can be exploited for developing elite culinary, ornamental, medicinal and industrial crops and their conservation.

Key words: *Ocimumbasilicum*,*Ocimumtenuiflorum*, genetic variability, RAPD, PCoA

Introduction

*Ocimum*Linn. Lamiaceae, collectively called basils, has long been acclaimed for its diversity. *Ocimum*comprises more than 30 species of herbs and shrubs fromthetropicaland subtropicalregions of Asia, Africa, Central and South America, but the main center of diversity appear tobe Africa (Paton, 1992). The genus is a source of essential oils and aroma compounds (Simon *etal*., 1984, 1990), a culinary herb, and an attractive, fragrant ornamental (Morales and Simon,1996; Morales *et al*., 1993). The seeds contain edible oils and a drying oil similar to linseed (Angers *et al*., 1996). The purple basils contained very high concentration of anthocyanins and may serve as a potential new source of stable red pigments for the food industry (Simon *et al*., 1999). Extracts of the basils are used in traditional medicines, and have been shown to contain biologically active constituents that are insecticidal, nematicidal, fungistatic, or antimicrobial (Deshpande *et al*., 1977; Chatterje*et al*., 1982; Albuquerque, 1996; Oxenham*et al*., 2005). Most commercial basil cultivars available in the market belong to the species *O. basilicum*. Darrah (1974, 1980)classified the *O. basilicum* cultivars in seven types: (1) tall slender types, which include the sweet basil group; (2) large-leafed, robust types, including Lettuce Leaf also called 'Italian basil'; (3) dwarf types, which are short and small leafed, such as 'Bush basil'; (4) compact types commonly called 'Thai basil'; (5) the purple-colored basil types with traditional sweet basil flavour (6) purple types such as 'Dark Opal', a possible hybrid between *O. basilicum* and *O.forskholei*, which has lobed-leaves, with a sweet basil plus clove-like aroma; and (7), lemon-flavored basils.

Tulsi or Sacred Basil (*Ocimumtenuiflorum*Linn. syn*Ocimum sanctum*Linn.), the legendary—Incomparable One, is one of the holiest and

most cherished of the many healing and health-giving herbs of the Orient. The common Indian varieties of Tulsi are 'Sri tulsi' or Lakshmi tulsi or 'green tulsi' with green stem and leaves with white inflorescences and black or 'purple tulsi' or 'Krishna tulsi' with purple stem, leaves and inflorescences. A morphotype 'pink' with pale purplish stem, green leaves, white inflorescences and pink flowers is also occasionally found. The healing benefits of the tulsi varieties are mostly similar, although some believe Krishna tulsi to contain slightly more healing potential. The study of genetic variability can contribute valuable guidelines for identifying genotypes with more commercial potential or medicinal strength and efficacy apart from conservation strategies of the species.

Various types of DNA markers were applied in basil genetic diversity studies including AFLP (amplified fragment length polymorphism), random amplified polymorphic DNA (RAPD), Inter-simple sequence repeat (ISSR), simple sequence repeats (SSR), sequence-related amplified polymorphism (SRAP) and also plastid and nuclear DNA sequence markers. For example, a genetic analysis of *O.basilicum* was carried out by Vieira *et al*. (2000, 2003) based on RAPD markers and volatile oil constituents. They found close relation between *O.basilicum* and *O.tenuiflorum*. Labra *et al*. (2004) conducted morphological characterization, analyzed essentialoil composition and DNA genotyping (AFLP) of *Ocimumbasilicum* L. cultivars. In another study of the genetic diversity and phylogeny of basils and allies Paton *et al*. (2004) studied the *trnL*intron,*trnL-trnF*intergenic spacer and*rps*16 plastid sequences of *O. basilicum*and *O.citriodorum*. Six different species of*Ocimum*from northern India were taken for studyinggenetic relationship using RAPD, ISSR and SSR markers by Lal et al. (2012). Chen et al. (2013) studied the genetic diversity among four species of *Ocimum* including *O. basilicum* and *O. tenuiflorum*from Taiwan based on ISSR, RAPD and SRAP markers. Ibrahim et al. (2013)carried out genetic variability studies among three sweet basil (*Ocimumbasilicum* L.) varieties from Egypt based on morphological traits and RAPD markers. Patel *etal*. (2015) also from northern India analyzed the genetic diversity of five species of *Ocimum* including *O. basilicum* and *O. sanctum* based on RAPD and ISSR markers. There is a lack of information on the molecular characterization of the *Ocimum* species from southern India. The objective of the present study is therefore to investigate the genetic diversity within and among three different varieties each of*Ocimumbasilicum* L .and *Ocimumtenuiflorum* (L.) Keng.from South India. This approach will be useful to determine elite genotypes of these species for commercial exploitation and also genetic conservation.

Materials and methods

Sample collection

Three varieties each of *O. basilicum* and *O. tenuiflorum* were used for the present study. The *O. basilicum*varieties included purple basil or*purpurascens*type (*O.basilicum*L. var. *purpurascens*Benth.), a slender herb with nearly single racemes, narrow lanceolate leaves and agenerally purple-color, dwarf types namely *pilosum* type or bush basil (*O. basilicum* L. var. *pilosum*Benth.) which are short and small leafed with narrower racemes and much smallerfruiting calyces, and *citriodorum* type or lemon basil with lemon-flavoured leaves (*O. basilicum* var*citriodorum*Benth.syn*O. citriodorum* Vis (Fig. 1: A-C) with traditional sweet basil flavour. These were collected from different localities in Kerala and Karnataka. Materials of the three morphotypes of *O. tenuiflorum* namely Krishna Tulsi or purple tulsi, Sri tulsi or green tulsi and the pink tulsi(Fig. 1: D-F) were collected from different parts of Kerala, Tamil Nadu,Karnataka and Andhra Pradesh (Table 1). The plants were identified by comparing with the authentic samples in the herbarium of Botanical Survey of India, Southern Circle. Voucher specimens were deposited in the Herbarium (KUBH) of Department of Botany, University of Kerala. Voucher numbers are given in Table1. Fully open young leaves were collected for molecular experiments. The leaf samples were cleaned and stored at -20^0 C.

RAPD analysis

Total genomic DNA from the young leaves of all the populations was isolated using CetylTrimethyl Ammonium Bromide (CTAB) method with appropriate modifications (Murray andThompson, 1980). The precipitated DNA was collected and washed with 80% ethanol. The DNA was resuspended in 0.5 ml 1 x TE buffer (10mM Tris- HCl, 1mM EDTA, pH 8.0). After treatment with RNase the DNA concentration was measured spectrophotometrically at 260nm. PCR for RAPD analysis was carried out using 10-mer oligonucleotide primers (Biogene C series). Fifteen primers were used for PCR amplification (Table 2). RAPD assay was carried out in 25µl reaction mixture containing 2.5 µl 10x amplification buffer [100mM Tris-HCl pH 9.0, 1.5 mM MgCl2, 50mM KCl and 0.01% gelatin], 22 µM each of dATP, dGTP,

dTTP, dCTP 1U Taq DNA polymerase(Perkin Elmer, Norwalk Conn), 50ng of template DNA and 15 pMoles of primers in 19.5µl sterile water. The amplifications were carried out in a MJ Research Inc.PTC-100 Programmable Thermal Cycler. After the initial cycle of 4 min at 940C, 1 min at 360C and 2 min at 720C, 40 cycles of 1 min at 940 C, 1min at 360C and 2 min at 720C followed by 4 min extension at 720C were performed. Amplified products were analysed by a 1.2% agarose gel with ethidium bromide using 1x TBE (Tris-Borate-EDTA) buffer at 10V/cm2 for 3 h.

The data obtained from RAPD assay were analyzed using WINBOOT software (Yap and Nelson, 1996) to estimate the genetic variability. The presence and absence of amplicons in the gels were scored as 0 and 1 respectively for the RAPD data analysis. Nei's original measures of genetic identity and genetic distance were calculated from this data. Cluster analysis using UPGMA (Sneath and Sokal, 1973) unweighted pair grouping method of averages), method was applied to estimate genetic distance values from Nei's genetic similarity values. The scored binary matrix was used to construct a dendrogram. Branch support was estimated using bootstrap analysis (Felsenstein, 1985). The following arbitrary scale for evaluating bootstrap (BS)was applied: weak (50%-74%), moderate (75%-84%) or strong (85%-100%).The Nei's similaritymatrix was used as the basis for ordination by Principal Component Analysis (PCA), which was performed to show the distribution of the genotypes in a scatter plot using the software MVSP version (Multivariate Statistical Package;http:/www. kovcomp.com/mvsp).

Results

RAPD analysis

The RAPD and PCR data of the two species are given in Table 2. A total of 168 bands were amplified 154 (91.67%) of which were polymorphic. Average number of bands per primer was found to be 11.2 and average number of polymorphic bands per primer was found to be 10.3. The number of amplification products generated by each primer varied from 6 to 14. The range of percentage polymorphism varied from 50% to 100%. All the 15 RAPD primers generated polymorphism and six primers could generate 100% polymorphism. The average value of polymorphism in *O. basilicum* analyzed individually was found to be 35.67% while that in *O.tenuiflorum*was 68.61%. Out of 15 primers analyzed two primers (C61 and C74) producedspecies- specific amplicons, one each in *O.tenuiflorum* Green morphotype. No species-specific alleles were detected in other populations of O. *tenuiflorum* or *O. basilicum*. Representative RAPD patterns generated in the two species by primers C61, C62, C63, C64, C65, C66, C67, C72, C74 and C75 are shown in Fig. 2: a- g.

Genetic similarity, cluster analysis and Principal components analysis

The similarity matrix obtained after multivariate analysis using Nei's coefficient is shown in Table 3. It revealed that overall genetic similarity between the populations of the two species is 0.67 and the similarity values ranged from 0.32(between R2 and B3) to 0.96 (between P2 and P3). The similarity coefficient values are used to generate a dendrogram by UPGMA analysis (Fig. 3) which clearly distinguished both the species. The dendrogram analysis indicated that the different populations formed two major clusters corresponding with the two species. Cluster I comprised populations of *O. basilicum* with strong branch support (BS= 100.0). This cluster represented the three different populations of *O.basilicum*. Cluster II with subgroups is formed from populations of *O. tenuiflorum* also with strong branch support(BS= 91.3- 100.0). In cluster I high genetic similarity is expected among the populations of *O.basilicum*. B2 and B3 were grouped together with a similarity coefficient of 0.52 and 0.53, and BS= 100.0. B1 was distinct from the other two with a similarity of 0.59 and BS= 99.9. In cluster II R1 and R2 appeared closely related at the genetic level with similarity values of 0.67 and 0. 62 and BS= 98.8. R3 in this cluster was distinct with only weak branch support. It grouped with P2, P3, G1, G2 and P1 with similarity values ranging from 0.72 to 0.76. G3 was found to be clustering separately from G1 and G2 with similarity value of 0.69 and weak branch support. Principal Component Analysis (PCA) was performed in order to determine the genetic relationships among the different populations. The result of PCA based on RAPD markers was comparable to the cluster analysis (Fig. 4). PCA data also indicated that the 12 populations comprise two different clusters delineating the two species. The genotypes belonging to a particular cluster were grouped together in the PCA plot. It showed that the *O.tenuiflorum*genotypes clustered together, whereas *O. basilicum*genotypes clustered as a secondgroup. The populations were plotted on principal coordinates 1 and 2, accounting for 44.28% and 15.11% of the variation respectively and together explaining 59.40% of the total variation. RAPD markers are thus found to be

well suited for determining the genetic diversity present in basil populations.

Discussion

Genetic variation governs the potential of a species to evolve and adapt. Little published information can be found about assessment of genetic diversity in *Ocimum* using a PCR-based approach (Lal *et al*. 2012; Chen *et al*. 2013). The intrinsic genetic diversity in the present study on *Ocimum* accessions was apparent from the analysis of their RAPD profiles and from the dendrogram generated where all the accessions had unambiguously separated from each other. Genetic analysis of the limited number of populations from different parts of South India using 15 RAPD primers in the present study was sufficient to bring out the relatively high level of genetic diversity in the two species. The level of polymorphism observed indicates a wide and diverse genetic base from different populations. The percentage of polymorphic bands (91.67%) was also higher. High polymorphism of RAPD markers was also reported in many previous studies, for example, RAPD of *Melocannabaccifera* (Lalhruaitluanga and Prasad 2009). The genetic similarity values generated out of the different accessions of *O.basilicum* during the present study ranged from 0.52 to 0.59 with an average G_{ST}value of 0.55 based on Nei and Li's similarity coefficient. The clustering of the three different populations together as a major cluster in cluster analysis with strong branch support can be attributed to the high genetic similarity index shared by them. Similarly the genetic similarity values in the different populations of *O.tenuiflorum* ranged from 0.69 to 0.76 with an average G_{ST} value of 0.71. Genetic variation at intraspecific level is a prerequisite for future adaptive change or evolution, and has profound implications for species conservation (Schaal*et al*., 1991). Coefficient of genetic differentiation, G_{ST} or if based on F-statistics, F_{ST}) is inversely proportional to genetic diversity i.e. high G_{ST} values indicate lower diversity due to population structuring (Luan et al., 2006). This may be attributed to phenomena like reproductive isolation, poor gene flow etc. Rate of gene flow (Nm) is directly proportional to genetic diversity. Therefore, a population with high genetic diversity will also have higher gene flow rate. In the present study, *O. basilicum* has low G_{ST} (0.55) while genetic diversity (GD) is relatively higher (GD= 1-0.55= 0.45) with higher gene flow rate. *O. tenuiflorum*with comparatively high G_{ST} (0.71)is genetically less diverse ((GD= 1-0.71= 0.29) with poor gene flow rate. The clustering of the nine different populations together as another major cluster with strong branch support in cluster analysis with subgroups can be attributed to the high genetic similarity index shared by them although geographically they are from different locations in south India (Table 1).

The 68.61% of genetic differentiation coefficient in O. *tenuiflorum* compared to the 35.87% of genetic differentiation coefficient in *O.basilicum* from RAPD analysis suggests that the species *O.tenuiflorum*exhibits a higher genetic diversity among the different populations than the otherspecies. Similar results have been reported in several plants (e.g. Rakhee*et al*., 2004) and animals (e.g. Sharma *etal*., 2003). So this technique provides information regarding inter and intra-specific variations. Principal component analysis was used to illustrate the multiple dimensions of the distribution of the genotypes in a scatter- plot. This multivariate approach was used to complement the information obtained from the cluster analysis methods because it is more informative regarding distances among major groups (Taran et *al*., 2005).

In the present study maximum similarity was observed between populations P2 and P3, and B2 and B3 with the values 0.96 and 0.95 respectively which is possibly due to high level of gene flow. As these populations are collected from highly distant localities compared to other populations it is seen that levels of gene flow are not directly dependent on distance but on other factors (Gibbs, 2001)

The different populations under the present investigation show seed set, though they are propagated naturally through vegetative means. Hence the polymorphism observed in the species may be an adaptation gained during evolution to acclimatize at the various geographical conditions. The diversity in basil based on appearance, flavors, fragrances, industrial, edible, and drying oils, and natural pigments (Phippen andSimon, 1998) as correlated with genetic diversity observed during the present study offers a wealth of opportunities for developing new culinary, ornamental, medicinal and industrial crops.

Conclusion

The high level of genetic polymorphism revealed by RAPD analysis in natural populations of O.*basilicum* and *O.tenuiflorum* might play a role in the evolution of the two species in southern India. Apart from conservation strategies of the species the present study may contribute a wealth

of opportunities for developing better varieties of the two species.

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Table 1
Details of genotypes ofOcimumbasilicumandO.tenuiflorumusedin the present study

	Name of accession	Place of collection	GPS location
O. basilicum			
1.	B1 purple basil 7004	Palode,Kerala	8°42′12″N 77°01′35″E
2.	B2 lemon basil 7002	Coorg,Karnataka	12.3375° N, 75.8069°E
3.	B3 bush basil 7005	Kalpetta,Kerala	11°36′18″N76°04′59″E
*O. tenuiflorum*purple 7010			
4.	R1	Trivandrum, Kerala	8.5241° N, 76.9366° E
5.	R2	Munnar,Kerala	10°05′21″N77°07′00″E
6.	R3	Coorg, Karnataka	12°3375″N75°8069″E
*O.tenuiflorum*green7007			
7.	G1	Trivandrum, Kerala	8.5241° N, 76.9366° E
8	G2	Coorg, Karnataka	18°20′00″N82°52′00″E
9.	G3	Visakhpatnam, AP	17.6868° N, 83.2185°E
*O.tenuiflorum*Pink 7009			
10	P1	Madurai, TN	9.9252° N, 78.1198° E
11	P2	Visakhapatanam,	17.6868° N, 83.2185°E
12	P3	Coorg,Karnataka	18°20′00″N82°52′00″E

Table 2.
List of primers and RAPD data in O. basilicumandO. enuiflorumused in the present study

Primers	Sequence 5′- 3′	No. of bands produced	No. of polymorphic bands
C 61	TTC GAG CCA G	11	10
C 62	GTG AGG CGT C	13	13
C 63	GGG GGT CTT T	9	9
C 64	CCG CAT CTA C	12	12
C 65	GAT GAC CGC C	11	9
C 66	GAA CGG ACT C	12	12
C 67	GTC CCG ACG A	11	10

C 72	TGT CAT CCC C	13	12
C 73	AAG CCT CGT C	6	5
C 74	TGC GTG CTT G	10	9
C 75	GAC GGA TCA G	8	4
C 76	CAC ACT CCA G	14	13
C 77	TTCCCCCCAG	14	14
C 78	TGAGTGGGTG	13	13
C 79	GTTGCCAGCC	11	9
Total No. of bands		168	154
Average/primer		168/15=11.2	154/15=10.3

Table 3.
Similarity matrix ofO. basilicum and O. tenuiflorumpopulationsbased on Nei' s

coefficient												
R1	1.00											
R2	0.84	1.00										
R3	0.76	0.75	1.00									
P1	0.67	0.59	0.79	1.00								
P2	0.73	0.65	0.85	0.87	1.00							
P3	0.72	0.64	0.85	0.85	0.96	1.00						
G1	0.74	0.65	0.85	0.89	0.91	0.89	1.00					
G2	0.73	0.65	0.84	0.79	0.86	0.89	0.89	1.00				
G3	0.69	0.59	0.74	0.80	0.78	0.77	0.83	0.82	1.00			
B1	0.50	0.45	0.50	0.54	0.52	0.54	0.53	0.56	0.54	1.00		
B2	0.35	0.33	0.36	0.44	0.43	0.42	0.44	0.43	0.36	0.76	1.00	
B3	0.34	0.32	0.40	0.44	0.44	0.45	0.46	0.44	0.37	0.74	0.95	1.00
	R1 R2		R3	P1	P2	P3	G1	G2	G3	B1	B2	B3

Fig. 1 A. *Ocimumbasilicum* var. *purpuracens* (purple basil) B. *O.basilicum*var.*pilosum* (bush basil) C. *O.basilicum* var. *citriodorum* (lemon basil) D. *O.tenuiflorum* purple type (Krishna tulsi) E. O. *tenuiflorum*green type (Sri tulsi) F. *O.tenuiflorum* purple green or pink type

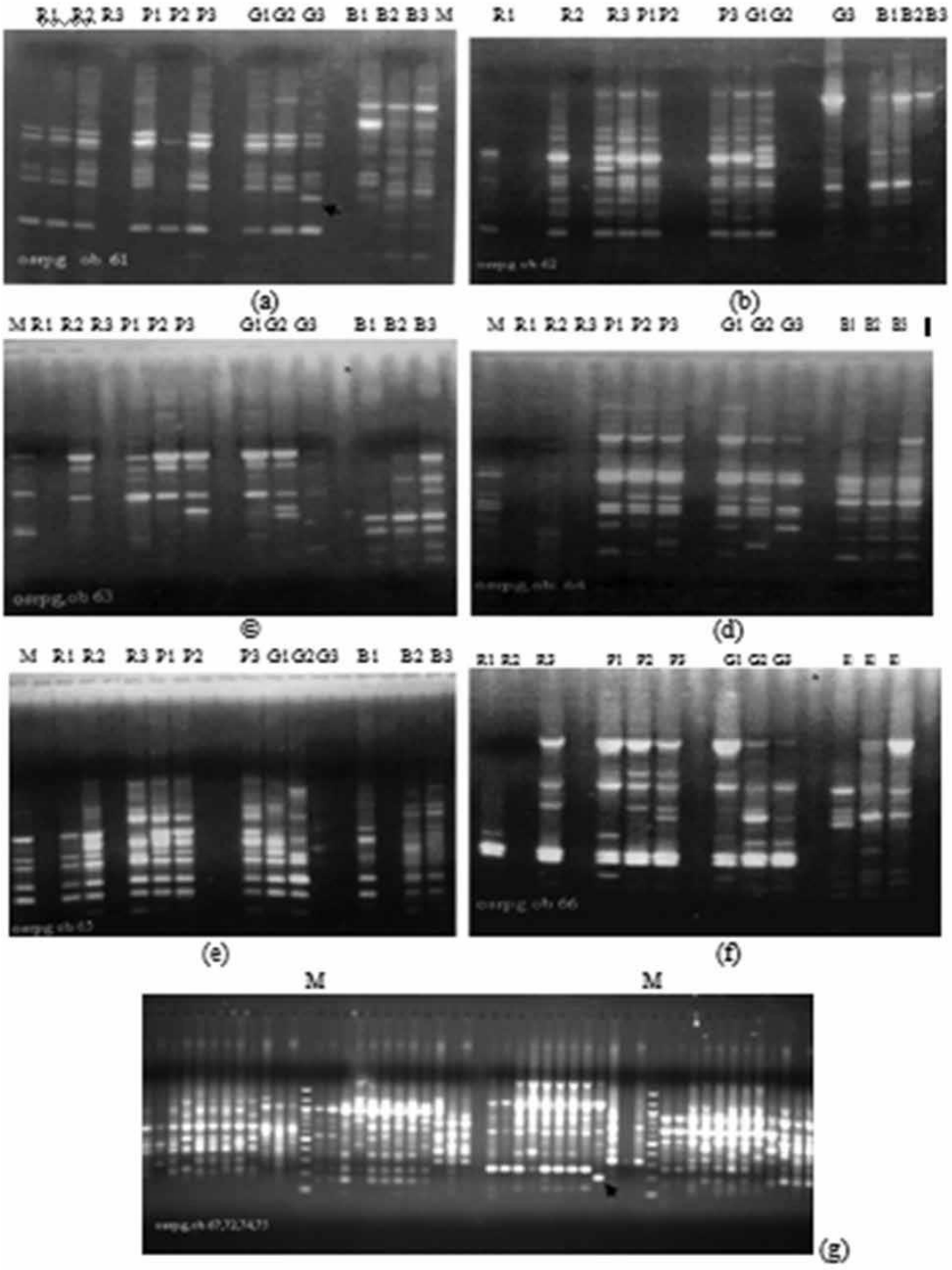

Fig.2 a-g: RAPD Profile of *O.basilicum* and *O.tenuiflorum*populations.The black arrows point towards the species – specific amplicons in *O.tenuiflorum* green type

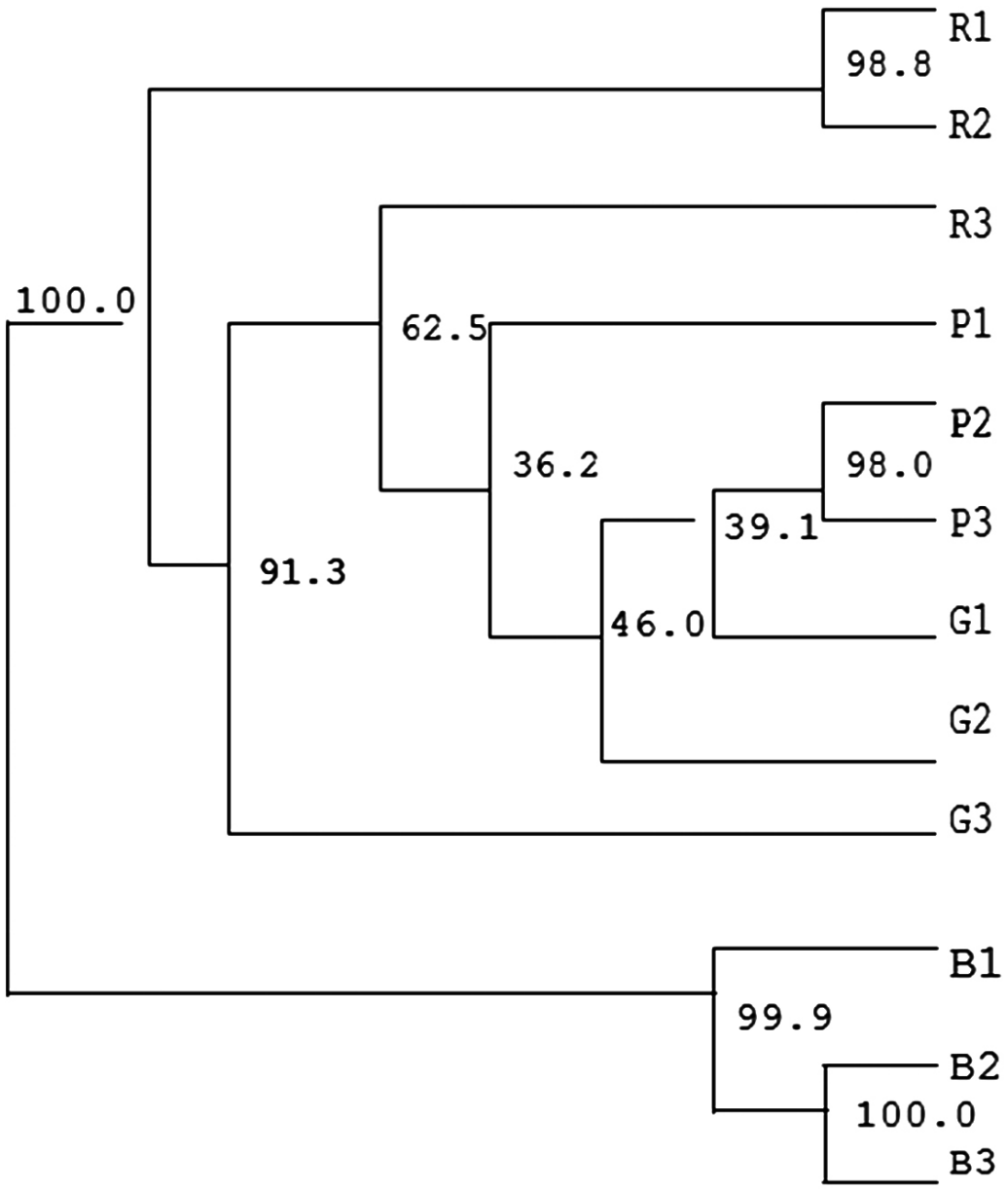

Fig.3 Dendrogram of Ocimum species showing diversity of populations of *O. basilicum* and *O. tenuiflorum*based on UPGMA analysis. Bar on the bottom represents similarity index basedon Nei's coefficients. Symbols on right indicate population code given in Table 1(B1-B3 *O. basilicum*populations; G1-3, P1-3 and R1-3 are green, pink and purple morphotypes of *O.tenuiflorum*)

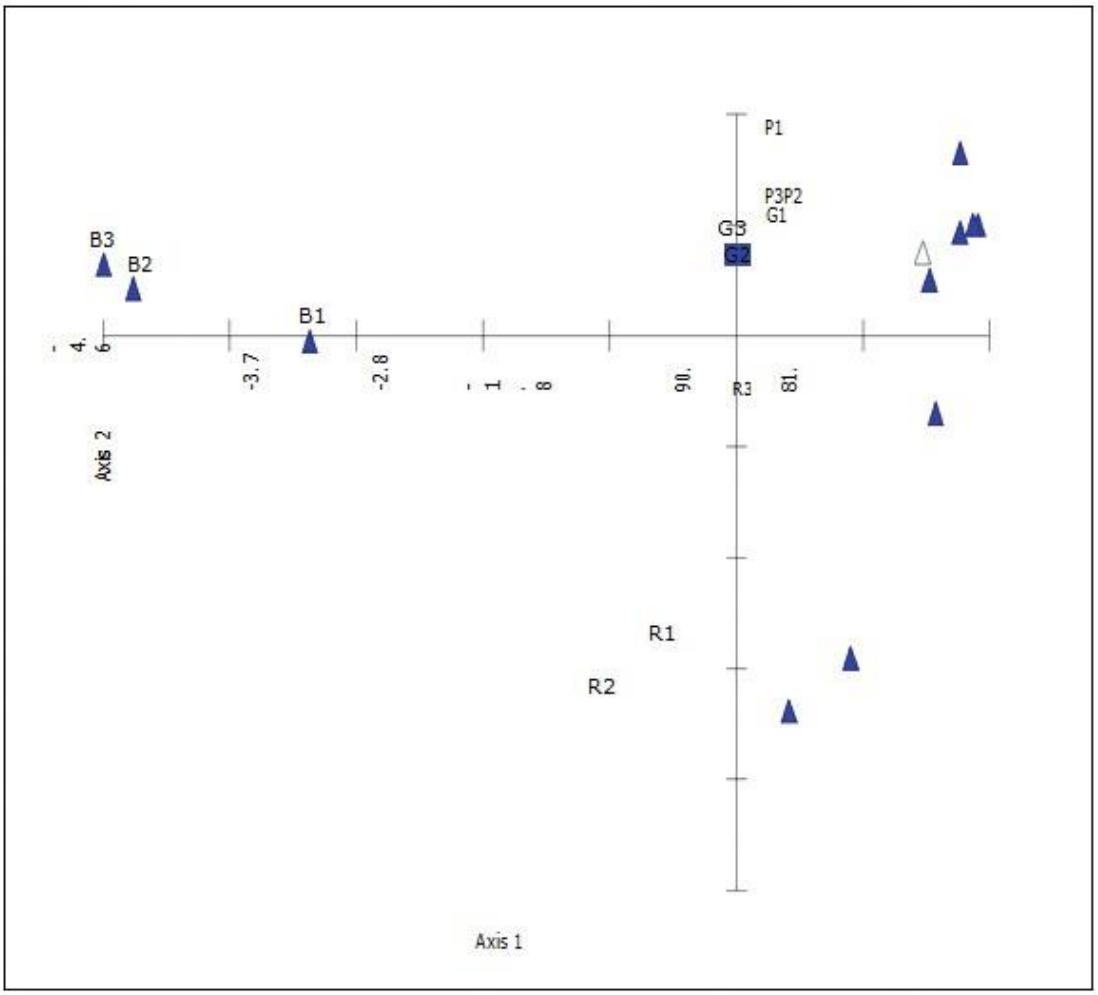

Fig. **4** Principal component analysis of 3 populations of *O.basilicum* and 9 populations of *O. tenuiflorum*based on Nei's similarity matrix. Symbols in the plotrepresent populations listedin Table 1 ((B1-B3 *O.basilicum* populations; G1-3, P1-3 and R1-3 are green, pink and purple morphotypes of *O.tenuiflorum*)