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Genetic diversity assessment in *Jasminum* species using Amplified Fragment Length Polymorphism

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Abstract

Jasmines (*Jasminum sp.*), a native of tropical and subtropical region, are esteemed for their attractive fragrant flowers and essential oil. However, very meagre information is available on the genetic relatedness among species and cultivars of jasmine. This study analyzed genetic relatedness of 48 genotypes across 26 species using Amplified Fragment Length Polymorphism markers. Of the ten sets of primers screened, four sets were selected for the present investigation on genetic diversity. A total of 212 bands were scored, of which 90.5% were polymorphic. The relationship among genotypes was analyzed using Unweighted Pair Group method of cluster analysis. Among 48 genotypes *J. auriculatum* formed a separate node while rest of the 47 genotypes formed two major clusters with two sub clusters in each. Cluster I comprised of 28 genotypes and cluster II had 19 genotypes. It was difficult to classify the genotypes either based on geographical region or based on their cultivation since there was assortment of wild and cultivated species. However the results reflect a high level polymorphism suggesting occurrence of genotypes of diverse genetic background that will be useful in breeding programmes.

Key words: Genetic diversity, *Jasminum*, AFLP, wild, cultivated

Introduction

A native of tropical and subtropical region, belonging to family Oleaceae, jasmines are esteemed for their attractive fragrant flowers and is highly valued for the essential oil. The species *J. sambac, J. grandiflorum* and *J. auriculatum* are commercially cultivated in many parts of India and contribute substantially to the national economy. Of late *J. multiflorum* and *J. angustifolium* are also being grown commercially and seen in substantial quantities in the flower market. However the flowers are not scented and hence are grown only for loose flowers and not for essential oil extraction.

The natural oils of jasmine are used for manufacturing high-grade perfume. The odour of jasmine flowers is unique that it cannot be imitated by any known synthetic aromatic chemical or natural isolates and thus giving it a unique status in the perfume world. Among Indian jasmines, scented species such as *J. grandiflorum, J. auriculatum, J. sambac, J. angustifolium, J. officinale, J. humile* and *J. pubescence* are found useful. Some high yielding varieties have been evolved which have high oil content and recovery also. Many eco-types are also found under cultivation (Bose *et al*., 2003).

The distribution of *Jasminum* genus is pan-tropical but a large number of species are centred around India, China and Malaysia (The Wealth of India, 1959). This comprises of diploids, triploids and tetraploids with n=13. Diploids (2n=26) are generally common in this genus. However natural occurrence of higher ploidy levels were also observed with *J. sambac cv.* Gundumallai (2n=39), *J. flexile* (2n=52), *J. primulinum* (2n=39) and *J. angustifolium* (2n=52). It is reported that this genus comprises of more than 200 species (Rendle, 1925; Bailey, 1958; Dickey, 1970) of which many are synonyms and 89 species are considered to be true in existence (Veluswamy, *et al.,* 1975) as indicated by Baker (1877), Cooke (1905) and Duthie (1911). Ambiguity exists regarding the occurrence of true number of species and genetic relationship because of synonyms in jasmine germplasm. This has led to difficulty in utilization of resources and breeding new varieties.

Amplified fragment length polymorphism markers have been employed widely to study genetic diversity in many horticultural crops. It has been used to study the genetic diversity and relatedness among wild and cultivated olive germplasm (Angiolillo, *et al.,* 1999; Belaj, *et al*., 2004; Baldoni *et al.,*2006; Sanz-Cortes, *et al*., 2003; Owen *et al*., 2005; Casas *et al.,* 2006; Rao *et al.,* 2009), in coffee (Coulibay *et al.,* 2003) and in *Osmanthus fragrans*, a traditional horticultural plant of Oleaceae valued for beauty and fragrance (Yuan *et al.,* 2008). Use of such molecular markers for genetic diversity studies in jasmine is very meagre. In the study reported here, we have assessed the genetic diversity among 48 genotypes belonging to 26 species of jasmine using AFLP markers.

Materials and methods

Plant material: Forty eight genotypes belonging to 26 species were collected during 2008-2012 from the states of Karnataka and Tamil Nadu in India (Table1) and maintained at Agricultural College, Hassan campus, Karnataka, University of Agricultural Sciences, Bangalore, India. Some of the species have been collected from their natural habitat of Western Ghats of Hassan, Chikkamagalur, Shimoga districts and also Dakshina Kannada districts of Karnataka state. Different variants within the species are named after the locality from where they have been collected.

Table 1. List of genotypes used for molecular characterization

Genotype	Speices	Region	Wild/Cultivated
1	*J. sambac* 1	Tamilnadu	Cultivated (Commercial)
2	*J. sambac* 2	Tamilnadu	Cultivated (Commercial)
3	*J. sambac* 3	Karnataka	Cultivated (Home garden)
4	*J. officinale*	Karnataka	Wild
5	*J. sambac* 4	Karnataka	Cultivated (Home garden)
6	*J. sp*1,Bangalore	Karnataka	Wild
7	*J. sambac* 5	Karnataka	Cultivated (Commercial)
8	*J. grandiflorum*1	Tamilnadu	Cultivated(Commercial)
9	*J. sambac* 6	Karnataka	Cultivated (Home garden)
10	*J. flexile*	Karnataka	Cultivated (Home garden)
11	*J. grandiflorum*2	Tamilnadu	Cultivated(Commercial)
12	*J. sambac* 7	Karnataka	Cultivated (Home garden)
13	*J. sp*2, Bangalore	Karnataka	Wild
14	*J. cuspidatum*	Karnataka	Wild
15	*J. multiflorum*1	Karnataka	Cultivated(Commercial)
16	*J. auriculatum*1	Tamilnadu	Cultivated (Commercial)
17	*J. mesni*	Karnataka	Cultivated (Home garden)
18	*J. rigidum*	Karnataka	Cultivated (Home garden)
19	*J. sambac*8	Karnataka	Cultivated (Commercial)
20	*J. sambac*9	Karnataka	Cultivated (Commercial)
21	*J. sambac*10	Karnataka	Cultivated (Home garden)
22	*J. sambac*11	Karnataka	Cultivated (home garden)
23	*J. sambac* 12	Karnataka	Cultivated (Commercial)
24	*J. sp*3Lalbagh	Karnataka	Wild
25	*J. primulinum*	Karnataka	Cultivated (Home garden)
26	*J. dichotomum*	Karnataka	Wild
27	*J. angustifolium*	Karnataka	Cultivated (Commercial)
28	*J. communis*	Karnataka	Wild
29	*J. sp* 4 Lalbagh	Karnataka	Wild
30	*J. sp* 5 Lalbagh	Karnataka	Wild
31	*J. sp* 6 Lalbagh	Karnataka	Wild
32	*J. auriculatum*2	Karnataka	Cultivated (Commercial)
33	*J. roxburghianum*	Karnataka	Wild
34	*J. sp* 7 Shimoga	Karnataka	Wild
35	*J. sp* 8 Puttur	Karnataka	Wild
36	*J. sambac* 13	Karnataka	Cultivated (Home garden)
37	*J. malabaricum* 1	Karnataka	Wild
38	*J. malabaricum* 2	Karnataka	Wild
39	*J. malabaricum* 3	Karnataka	Wild
40	*J. multiflorum* 2	Karnataka	Cultivated (Commercial)
41	*J. calophyllum*	Karnataka	Cultivated (Home garden)
42	*J. multiflorum*3	Karnataka	Cultivated (Commercial)
43	*J. multiflorum* 4	Karnataka	Cultivated (Commercial)
44	*J. multiflorum* 5	Karnataka	Cultivated (Commercial)
45	*J. multiflorum*6	Karnataka	Cultivated (Commercial)
46	*J. rottlerianum*	Karnataka	Wild
47	*J. sambac* 14	Karnataka	Cultivated (Home garden)
48	*J. ritchiei*	Karnataka	Wild

The genotypes include both wild and cultivated types that are grown either on a commercial scale or in home gardens.

DNA isolation: Total genomic DNA was extracted from the leaf samples collected from the new flushes. One gram of immature leaf tissue, collected from new shoots was washed using distilled water, wiped and used for DNA extraction. Extraction of DNA was performed by CTAB method (Porebski *et al*., 1997) with minor modifications.

Fresh leaf samples were powdered in liquid nitrogen and mixed with 10 mL of preheated extraction buffer containing 100mM Tris HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% (w/v) CTAB and 1% Polyvinyl pyrrolidone and 1% β-mercaptoethanol. The contents were mixed slowly and were incubated in water bath at 60 °C for 30 min with intermittent shaking. After incubation the contents of the tube were cooled to room temperature and equal volume of chloroform and Isoamyl alcohol (24:1) mixture was added. The tube was then gently inverted to mix the contents and centrifuged at 9500 rpm for 10 min at room temperature. The supernatant was collected in a separate tube and 0.7 volume ice cold Isopropanol was added to precipitate the DNA. Precipitation of DNA was improved by keeping overnight at -20 °C. The next day sample was centrifuged at 9500 rpm for 30 min to collect the pellet. The DNA pallet was then washed with 2-3 mL of 70% ethanol, air dried and dissolved in 1 mL TE buffer. The sample was added with RNAase (10µg/µL) and incubated at 37°C for 1h to remove RNA.

Equal volume of 1:1 Chloroform: Isoamylalcohol (24:1) was added to this and centrifuged at 9500 rpm for 10 min. The aqueous phase was collected and the step was repeated 2-3 times. To this 1/10th volume of 3M sodium acetate pH 5.2 and 2 volumes of ethyl alcohol (70%) was added and kept at 0 °C for half an hour. It was then centrifuged at 13000 rpm for 15 min to pellet the DNA. The supernatant was discarded the pellet was dried. The pellet was dissolved in 100 µL of TE buffer (10:1) and stored at 4 °C. The DNA was quantified using Nanodrop 2000 and verified by electrophoresis on 0.8% agarose gels.

AFLP analysis: AFLP analysis followed by silver staining was performed as described by Vos *et al.* (1995). The enzymes *EcoRI, MseI* and *T_4 DNA ligase* were purchased from New England Biolabs (UK) Ltd. The adaptors and primers were purchased from Sigma-Aldrich, Bangalore, India. Polymerase Chain Reaction was carried out in Eppendorf Master Cycler Gradient, Eppendorf India. The genomic DNA (0.5 µg) of all the 48 genotypes were double digested with 10 units of *EcoRI* and 4 units of *MseI* at 37 °C for 3 h in the presence of 1x restriction buffer purchased from New England Biolabs, (UK) Ltd. in 50 µL at 37 °C for 3 h. The digestion was followed by ligation of 5 pmol *EcoRI* adapters and 50 pmol of *MseI* adapters in the presence of 1 unit of T_4 DNA ligase and 1 mM ATP in a final volume of 10 µL. The digestion was carried out at room temperature over night. In preliminary experiments three dilutions of restricted/digested DNA (1:1, 1:5 and 1:10) in TE (10 mM Tris-HCl and 1 mM EDTA) buffer were tested for pre selective amplification and selective amplification where 1:1 dilution was found optimum. Pre selective amplification was carried out with primers carrying one selective nucleotide involving twenty cycles at 94 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s followed by 10 °C for 30 min in an Eppendorf Master Cycler. Pre amplification products diluted to 1:1 were used as template for selective amplification consisting of ten different primer pairs with three selective nucleotides (Table 2). Eleven cycles of 94 °C for 30 s, 65 °C for 30 s reducing by 0.7 °C each subsequent cycle to 56 °C and 72 °C for 60 s followed by twenty four cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s. The samples were denatured by adding 8 µL of stopping loading dye (98% formamide, 10mM EDTA pH 8, 0.05% bromophenol blue and 0.05% xylene cyanol) and heated at 94 °C for 5 min to cause denaturation and then cooled to 10 °C for 5 min. The product was finally stored at -20 °C. Three µL of each sample was run in 6% denaturing polyacrylamide gel (1x TBE buffer) in a sequencing gel electrophoresis unit at 1600 V for 1.5 h. Amplified fragment

Table 2. Oligonucleotide adaptors and primers used for AFLP analysis

Name	Sequences
*Eco*RI adaptor	5'-CTCGTAGACTGCGTACC- 3'
	3'-CTGACGCATGGTTAA-5'
MseI adaptor	5'-GACGATGAGTCCTGAG-3'
	3'-TACTCAGGACTCAT-5'
Primers used in preamplification	
EcoRI+A	5'-GACTGCGTACCAATTC+A-3'
MseI+C	5′-GATGAGTCCTGAGTAA+C-3′
Primers used in selective amplification	
EcoRI+AAC	5'-GACTGCGTACCAATTC + AAC-3'
MseI+CTT	5′-GATGAGTCCTGAGTAA + CTT-3′
EcoRI+ACC	5'-GACTGCGTACCAATTC + ACC-3'
MseI+CAT	5′-GATGAGTCCTGAGTAA + CAT-3′
EcoRI+ACT	5'-GACTGCGTACCAATTC + ACT-3'
MseI+CTG	5′-GATGAGTCCTGAGTAA + CTG-3′
EcoRI+ATG	5'-GACTGCGTACCAATTC + AAG-3'
MseI+CTC	5′-GATGAGTCCTGAGTAA + CTC-3′
EcoRI+AAG	5'-GACTGCGTACCAATTC + ACT-3'
MseI+CTT	5′-GATGAGTCCTGAGTAA + CTT-3′
EcoRI+ACT	5'-GACTGCGTACCAATTC + ACT-3'
MseI+CAC	5′-GATGAGTCCTGAGTAA + CAC-3′
EcoRI+AAC	5'-GACTGCGTACCAATTC + AAC-3'
MseI+CTG	5′-GATGAGTCCTGAGTAA + CTG-3′
EcoRI+ACT	5'-GACTGCGTACCAATTC + ACT-3′
MseI+CTC	5′-GATGAGTCCTGAGTAA + CTC-3′
EcoRI+AAG	5'-GACTGCGTACCAATTC + AAG-3′
MseI+CTC	5′-GATGAGTCCTGAGTAA + CTC-3′
EcoRI+AAG	5'-GACTGCGTACCAATTC + AAG-3′
MseI+CTT	5′-GATGAGTCCTGAGTAA + CTT-3′

length polymorphism was visualized by staining the gel using silver staining technique.

Data analysis: Each gel showing clear and reproducible polymorphic bands were scored manually for the presence (denoted as 1) or absence (denoted as 0) of bands in individual lanes. Genetic similarity was calculated using NTSYS-pc version 2.1 (Rohlf, 2000). Cluster analysis was based on distance matrices using the Unweighted Pair Group Method of Arithmetic averages (UPGMA) and relationship between samples have been graphically represented as dendrogram.

Results and discussion

Forty eight genotypes representing 26 species of *Jasmine* were screened with 10 *EcoRI*/*MseI* primer combinations. Four primers that produced clear banding pattern with polymorphism were used to fingerprint the genotypes. The number or AFLP fragments per primer combination ranged from 47 *(EcoRI*+ACC/ *MseI*+CAT) to 57 (*EcoRI*+ACT/*MseI* +CTG and *EcoRI*+AAG/*MseI*+CTC). A total of 212 scorable AFLP bands were generated from four primer combinations with an average of 53 bands per primer combination. The average polymorphism noticed among the jasmine genotypes from four primer combinations was 90.5% (Table 3). The AFLP analysis successfully differentiated the

Table 3. Selective primer pairs and level of polymorphism from four primer combinations.

Primer pair	Total bands scored	Polymorphic Markers	Percentage (%)
EcoRI+ACC/MseI+CAT	47	42	89.30
EcoRI+ACT/MseI+CTG	57	53	92.90
EcoRI+AAG/MseI+CTC	57	52	91.20
EcoRI+AAG/MseI+CTT	51	45	88.20
Total	212	192	90.50

jasmine genotypes and suggested a high level of polymorphism among the genotypes.

AFLP has been employed in assessing genetic diversity in various horticultural crops. Rosa *et al.* (2003), Sensi *et al.* (2003), Baldoni (2006), Casas *et al.* (2006), Yuan *et al.* (2008) and Rao *et al.* (2009) have reported the genetic diversity in olives (Family Oleaceae) using AFLP. Varied number of AFLP bands have been reported in many perennial horticultural crops such as Cuban pineapple germplasm which produced 44.6 bands on an average (Ermis Yanes Paz *et al*., 2012) and *Cucurbita moschata* germplasm collections with 55.56 fragments (Junxin Wu *et al.*, 2011). Similarly very high levels of polymorphism is also reported in horticultural crops with as high as 99.3% in *Erianthus arundinaceus* (Cai *et al.*, 2012); 91.0% in 34 cultivars loquat (*Eriobotrya japonica*) (Wu *et al*., 2011) 95.35% in 58 jujube cultivars and 2 wild types of sour jujube (*Ziziphus acidojujuba*) (Wen *et al*., 2009) and 91.88 to 98.15 % in Omani banana cultivars (*Musa* cvs.) (Nadiya *et al.*, 2010).

Occurrence of 90.5% polymorphism in *Jasminum* species reveals abundant genetic diversity existing among the genotypes. Our study includes 26 species and intraspecific cultivars or varieties that have contributed to the high level of polymorphism.

Genetic relationship among 48 jasmine cultivars genotypes was analyzed by unweighted pair-wise method (UPGMA) using Jaccard's similarity coefficient of NTSYS pc 1.2 program. Highest genetic similarity was observed between *J. grandiflorum*1 and *J. sambac*6 (0.58) and the lowest similarity was between *J. officinale* and *J. species* Lalbagh which was as low as 0.11. These low values of genetic similarity may be due to inclusion of both cultivated and wild species in this study. The similarity within cultivated species such as *J. sambac, J. auriculatum, J. grandiflorum* and *J. multiflorum* was also very less. Similarity between fourteen variants of *J. sambac* ranged from 0.41to 0.17. Two variants each of *J. auriculatum* and *J. grandiflorum* also had very low similarity of 0.1 and 0.38, respectively. The values varied between 0.14 to 0.18 among six genotypes of *J. multiflorum.*

The UPGMA dendrogram derived from the similarity matrix resulted in *J. auriculatum*1 forming a separate node while rest of the 47 genotypes clustering together (Fig. 1). These 47 genotypes formed two major clusters, cluster I consisting of 28 genotypes and cluster II consisting of 19 genotypes.

In cluster I, the three genotypes *J. mesni, J. primulinum* and *J. sambac*11 grouped together and had highest genetic distance from the other members of this cluster. Both *J. mesni* and *J. primulinum* are grown in home gardens and they produce yellow coloured

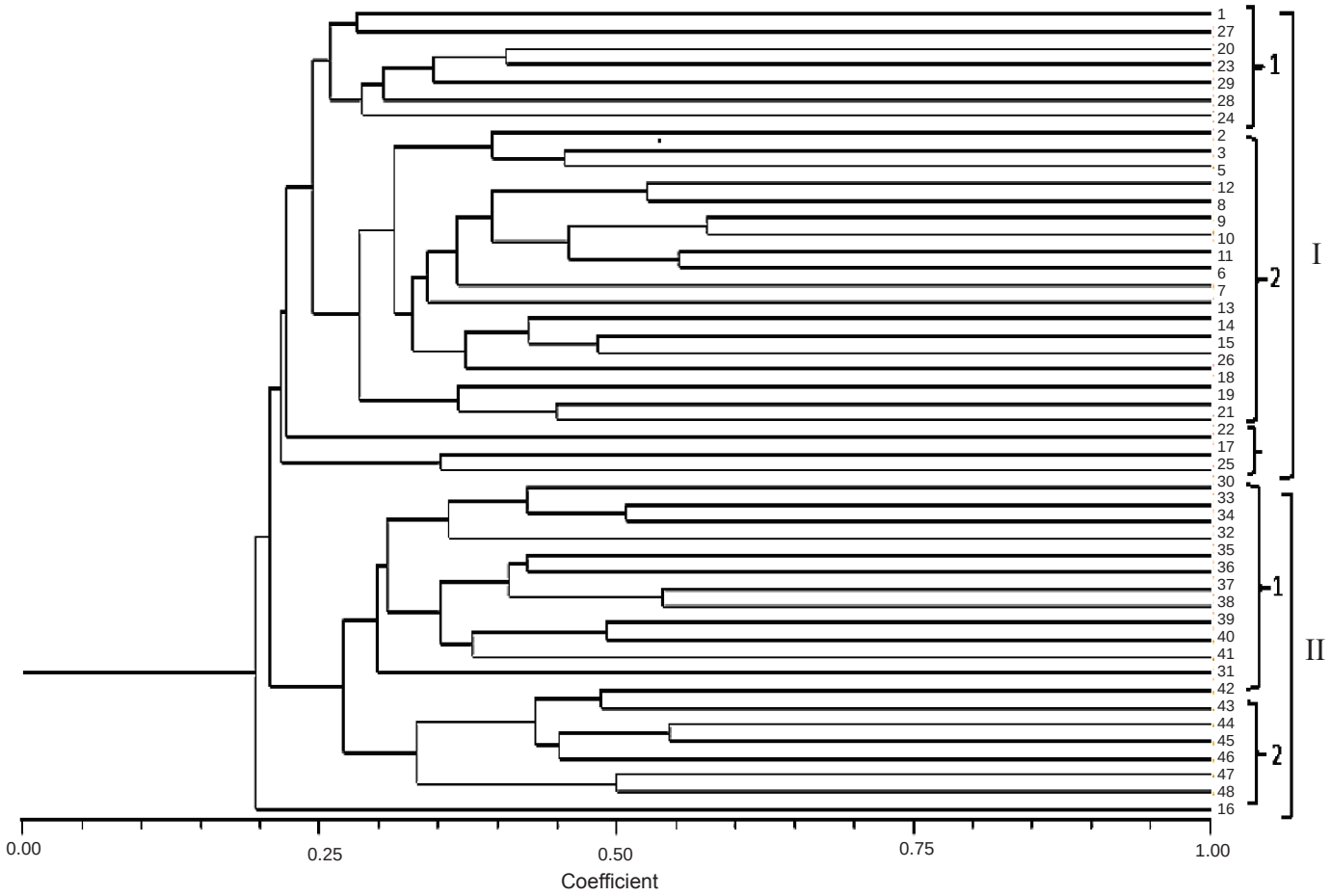

Fig.1. UPGMA based dendrogram showing the relationships among 48 genotypes of jasmines based on AFLP- based similarity coefficients

flowers unlike all other genotypes which produce white flowers or white petals with pink underneath. These yellow flowering genotypes had a similarity index of 0.35. The rest of the 25 genotypes of cluster 1 formed two sub clusters. Sub cluster 1 included all wild species except *J. sambac*1and *J. sambac*12 with 0.41 similarity. Sub cluster 2 consisted of majority of the cultivated species including eight of the 14 variants of *J. sambac*, two *J. grandiflorum* variants and *J. multiflorum*1 along with two species cultivated in home gardens (*J. officinale* and *J. flexile*) and five wild species dispersed in the cluster.

In cluster II, there were two subclusters in which subcluster 1 had nine wild species along with one genotype each from *J. auriculatum* (*J. auriculatum*2) and *J. sambac* (*J. sambac*13). Subcluster 2 had four genotypes of cultivated species *J. multiflorum* and one genotype of *J. sambac* (*J. sambac*14) along with two wild species *viz*., *J. rottlerianum* and *J. ritchiei.* These results indicate assortment of cultivated and wild genotypes of jasmine along with those grown in home gardens making it difficult to classify them either based on their geographical locations or based on their extent of cultivation or domestication. Many wild species share the same cluster with cultivated species indicating the probability of their relatedness in their origin and genetic background.

The cultivated jasmines are vegetatively propagated though some of the wild species or those found in home garden record very good seed setting. Due to poor seed setting and seed germination hybridization is rare among vegetatively propagated commercially important species. This may be the reason for majority of the *J. sambac* genotypes to cluster together in one group in sub cluster 2 of cluster 1.

In spite of high genetic diversity recorded in the present study, there is no clear grouping of the accessions from one single species. There is admixture of genotypes from wild and cultivated which includes both commercially cultivated and those grown in home gardens. High genetic dissimilarity was observed among the genotypes belonging to different species. This may be due to their relatedness in respect to their origin and exchange of genetic material due to hybridization. Such ambiguity in clustering have also been reported in olives. Belaj *et al.* (2007) examined the pattern of genetic variability and genetic relationships of 11 wild olive populations (171 individuals) olive (*Olea europaea* subsp. europaea var. sylvestris) in the north-western Mediterranean with eight microsatellite markers. The study was unable to identify clear-cut genetic boundaries between candidate areas containing either genuinely wild germplasm. Such high level genetic dissimilarity was noticed also in olives by Brito *et al.* (2008) in their analysis of native olive plants from *Olea maderensis* (*O. europaea* ssp. cerasiformis) and *O. cerasiformis* (*O. europaea* ssp. guanchica), wild olives (*O. europaea* ssp. europaea var. sylvestris) and cultivated olives (*O. europaea* ssp. europaea var. europaea) with respect to genome size and microsatellite markers. In the AFLP study by Angiolillo *et al.* (1999), the cultivated varieties of a single species *Olea europea* was compared with wild olives and also with a group of individuals belonging to different *Olea* species. They hypothesized that cultivars and wild plants are different forms of the same *O. europea* species. Sanz-Cortes *et al*. (2003) reported a high degree of diversity among the olive varieties present in each growing region. Genetic diversity studies

in jasmines using molecular markers are very few. Mukundan *et al.* (2008) noticed low to moderate genetic diversity among four south Indian *Jasminum* sp. using RAPD marker, the results of which is in contrast to our study as their investigation included varieties from only cultivated jasmines of South India.

The outcome of our study is useful in identifying diverse parental genotypes to create segregating progenies with maximum genetic variability for further selection in breeding programme. The desirable genes from the wild species and those grown in home gardens can be transferred to the genetic base of commercially cultivated species. The information on genetic diversity will help in germplasm identification, management and conservation.

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