Genetic diversity and genetic relationships in Hyacinthaceae in India using RAPD and SRAP markers

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Abstract Genetic diversity and relationship among three genera namely *Drimia*, *Dipcadi* and *Ledebouria* of Hyacinthaceae in India was studied using RAPD and SRAP markers. Twenty one RAPD primers and nine SRAP were used for analyzing 41 accessions. RAPD gave an average 12.6 markers per primer, while SRAP generated 10.1 markers per primer pair. The family emerged very diverged with high polymorphism. The study resolved the three genera into monophyletic groups corresponding to three subfamilies; Urginoideae, Hyacinthoideae and Ornithogaloideae. *Drimia wightii* emerged a very distinct species and species specific markers were obtained with both marker systems. AMOVA analysis also revealed the genera to be quite well diverged. The two markers showed high correlation ($r=0.932$) in Mantel matrix cresspondance test. The combined data also showed a very good correlation with the respective markers individually.

Keywords *Drimia* · *Dipcadi* · Genetic diversity · SRAP · RAPD

Introduction

Hyacinthaceae in India is represented by three genera *Drimia* Jacq. ex. Willd (syn. *Urginea* Steinh.), *Dipcadi* Medik. and *Ledebouria* Roth. These are small perennial bulbous herbs. The three genera have been subjected to taxonomic disputes at

interspecific levels and have been revised from time to time (Deb and Dasgupta 1981, 1987; Kartikeyan et al. 1989; Dixit and Yadav 1989; Pfosser et al. 2003). *Drimia* has six species in India viz. *D. coromandaliana* [syn. *U. coromandaliana* (Roxb.) Hook. f.], *D. indica* (Roxb.) Jessop, *D. polyantha* (Blatt. and McC.) Anand Kumar, *D. polyphylla* (Hook. f.) Ansari et Raghavan, *D. razii* Ansari and *D. wightii* Laksmin. *D. coromandaliana* is a tetraploid and considered to be autotetraploid of *Drimia indica* (Naik 1973, 1976) whereas others consider it a distinct species with segmental polyploid nature on the basis of cytological, palynological and hybridization studies (Dixit and Yadav 1989). *Drimia indica* has several cytotypes of different ploidy level. The genus *Dipcadi* is represented in India by seven species viz. *Dipcadi concanense* (Dalz.) Baker, *Dipcadi erythreum* Webb. Et Berth., *Dipcadi minor* Hook. f., *Dipcadi montanum* (Dalz.) Baker, *Dipcadi saxorum* Blatter, *Dipcadi serotinum* (L.) Medik. and *Dipcadi ursulae* Blatter (Deb and Dasgupta 1981). These species have several overlapping morphological characters, making species delimitation difficult. *Ledebouria* is reported to have three species; *L. hohenackeri* (syn. *Scilla hohenackeri* Fisch. & Mey.), *L. hyacinthina* Roth. and *L. viridis* (syn. *Scilla viridis* Salisb.) in India, however, the former one species has been reported only from their type collections and have never been collected thereafter. *Ledebouria hyacinthina* has diploid, triploid and tetraploid cytotypes inhabiting different types of habitats distributed over central India and Deccan Plateau. This genus has high degree of multiformity in morphology. Diploids are widely distributed in major parts of India especially in the plains with rocky undisturbed terrains and reproduce sexually. Triploid form ($2n=45$) is restricted to Western Ghats and Malabar Coast with high rainfall area. Although it produces healthy inflorescences, seed setting is rare and it propagates by formation of bulbils at leaf tips. Tetraploid form ($2n=60$) is restricted to extremely dry regions and reproduces sexually.

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Most of the species of Hyacinthaceae in India are endemic to Western Ghats, a biodiversity hotspot and a world heritage site. They constitute important genetic resource. *Drimia* (syn. *Urginea*), commonly called Squill has medicinal importance and has been mentioned in ancient texts and pharmacopoeias. It possesses cardiogenic stimulant and is used in case of congestive heart failure. The cardiac action of the drug resembles that of digitalin. It is also used as expectorant and has diuretic properties. It is also reported to have insecticidal, antifungal, antiangiogenic and proapoptotic activity (Pascual-Villalobos 2002; Deepak and Salimath 2006).

Sequence Related Amplified Polymorphism (SRAP) is a marker system developed by Li and Quiros (2001). It is a PCR based marker system that uses two primers. The polymorphism results from two events, first, due to insertion and deletions which lead to co-dominant markers and second, due to nucleotide changes leading to dominant markers. SRAP is similar to RAPD but have preferential random amplification of coding regions in genome and is more reproducible. SRAPs targets ORF so that they have more direct relation with the functional genes and it gives an opportunity to infer the relationships based on expressed part of the genome. It has successfully been employed in genetic diversity, genetic relationship, mapping and gene tagging studies (Budak et al. 2004; Peng et al. 2008; Zhao et al. 2009; Fu et al. 2010; Wang et al. 2011; Huang et al. 2012). RAPD is simple and convenient and has been used for the evaluation of genetic diversity and genetic relationship along with SRAP markers (Li-Wang et al. 2008; Fu et al. 2010; Comlekcioglu et al. 2010). The objective of the present study is to (i) evaluate the genetic diversity, (ii) study the interspecific relationship in the genera of family Hyacinthaceae in India and (iii) to compare the two marker systems for their ability to study genetic relationship in the family Hyacinthaceae.

Material and methods

Plant material and DNA extraction

Forty one populations belonging to 13 species of 3 genera of Hyacinthaceae were collected from different parts of India (Fig. 1; Table 1). The leaves were collected in June–July when leaves are present as the plant remains dormant for rest of the year. The leaves were collected in the field and immediately frozen in Liquid Nitrogen and stored at -20°C till further use. The voucher specimens have been submitted in herbarium of Botany Department, Shivaji University. Genomic DNA was extracted and purified from leaves using CTAB method (Doyle and Doyle 1987) with some minor modifications. DNA was quantified using Dynaquant TM 200 fluorometer (Hoefer Instruments, USA) using DNA intercalating Hoechst

33258 dye (Brunk et al. 1979). The DNA samples were diluted to $10\text{ ng}/\mu\text{l}$ for PCR amplification.

RAPD amplification

Fifty-five random decamer primers including 20 primers from series E (Bangalore GeneI, India), 17 primers from OPA and 18 from OPB series (Operon Technologies, USA) were initially screened for amplification and polymorphism. Out of these primers, 21 primers were selected for studying the members of the family Hyacinthaceae on the basis of reproducibility (Table 2). Amount of template DNA and MgCl_2 to be used in the PCR was first optimized by using 2–30 ng DNA and 1.5–5.0 mM MgCl_2 in separate reactions. Finally, a 25 μl reaction mixture containing 15 ng DNA template, 1X *Taq* buffer, 1U *Taq* polymerase, 1.5 mM MgCl_2 , 2 μM primer and 0.2 mM each dNTPs was used for the PCR. The PCR amplification conditions were as follows: (i) Denaturation at 94°C for 3 min (ii) 40 cycles of (a) denaturation at 92°C for 1 min (b) annealing at 35°C for 1 min and (c) extension at 72°C for 1 min 30 s and (iii) final extension at 72°C for 10 min. The amplified products were electrophoresed on 1.8 % agarose, stained with ethidium bromide and viewed in UV and photographed in Gel Doc (Biorad, USA).

SRAP amplification

The 25 combinations of five forward and five reverse SRAP primers previously reported by Li and Quiros (2001) were screened for amplification in the members of Hyacinthaceae. Nine primer pairs giving reproducible amplification were finally used for analyzing the 41 populations (Table 3). The PCR mixture contained the components as described for the RAPD except 1 μM of each SRAP primer pair instead of 2 μM of random RAPD primers. The PCR conditions were as follows: denaturation at 95°C for 5 min followed by five cycles of (i) denaturation at 94°C for 1 min (ii) annealing at 38.5°C for 1 min and (iii) extension at 72°C for 2 min, then increasing the annealing temperature to 49°C for 35 cycles and finally extension at 72°C for 10 min. The amplified products were electrophoresed on 1.6 % agarose, viewed in UV and photographed.

Data analysis

The amplified fragments were scored manually for the presence (1) and absence (0) to create a binary data matrix. The data matrix was analyzed using NTSYS-pc software version 2.11 (Rohlf 1992). Pairwise similarity matrix was computed based on Jaccard (1908) according to $J(i_1i_2) = a/(a+b+c)$ where a =number of shared bands between both the individuals i_1 and i_2 ; b =number of bands exclusive to individual i_1 and c =number of bands exclusive to individual i_2 . Dendrograms were

Fig. 1 Map of India showing the sites of collection of 41 populations analyzed, belonging to three genera, viz., *Dipcadi*, *Drimia* and *Ledebouria* of family Hyacinthaceae

created by Unweighted Pair Group Method with Arithmetic Average (UPGMA) using SHAN (Sequential Hierarchical

Agglomerative and Nested) analysis. The confidence limits of the dendrogram were estimated by bootstrapping with

Table 1 List of 41 taxa of Hyacinthaceae in India analyzed in the present study with other details

S. No.	Taxa	Chromosome number	Area of collection	Code	Geographical location
1	<i>Dipcadi concanense</i> (Dalz.) Baker	2n=12	Ratnagiri, Maharashtra	DC-R	17° 05' N, 73°17' E
2	<i>Dipcadi erythreum</i> webb et Berth	2n=22	Jodhpur, Rajasthan	DE-J	26° 21' N, 72°97' E
3	<i>Dipcadi minor</i> Hook. f.	2n=20	Halkarni, Maharashtra	DMN-HL	15° 54' N, 74°17' E
4	<i>Dipcadi montanum</i> (Dalz.) Baker	2n=20	Ajara, Maharashtra	DM-AJ	16° 08' N, 73°49' E
5	<i>Dipcadi montanum</i> (Dalz.) Baker	2n=20	Nesri, Maharashtra	DM-N	16° 04' N, 74°19' E
6	<i>Dipcadi saxorum</i> Blatter	2n=12	Mumbai, Maharashtra	DSX-C	19° 08' N, 72°51' E
7	<i>Dipcadi serotinum</i> (L.) Medik.	2n=12	Delhi	DS-DL	28° 61' N, 77°17' E
8	<i>Dipcadi ursulae</i> Blatter	2n=20	Kas, Maharashtra	DU-K	17° 41' N, 73°49' E
9	<i>Dipcadi ursulae</i> Blatter	2n=20	Thoseghar, Maharashtra	DU-T	17° 44' N, 73°49' E
10	<i>Drimia wightii</i> Lakshmin.	2n=20	Kartikswami, Maharashtra	UC-KS	15° 55' N, 74°28' E
11	<i>Drimia wightii</i> Lakshmin.	2n=20	Nesri, Maharashtra	UC-N	16° 04' N, 74°19' E
12	<i>Drimia wightii</i> Lakshmin.	2n=20	Gokak, Maharashtra	UC-G	16° 10' N, 74°49' E
13	<i>Drimia coromandaliana</i>	2n=4x=40	Kagal, Maharashtra	UCR-K	16° 34' N, 74°18' E
14	<i>Drimia coromandaliana</i>	2n=4x=40	Appachivadi, Maharashtra	UCR-A	16° 29' N, 74°18' E
15	<i>Drimia indica</i> (Roxb.) Jessop	2n=20	Delhi	UI-DL	28° 61' N, 77°17' E
16	<i>Drimia indica</i> (Roxb.) Jessop	2n=20	Jodhpur, Rajasthan	UI-J	26° 21' N, 72°97' E
17	<i>Drimia indica</i> (Roxb.) Jessop	2n=20	Pushkar, Rajasthan	UI-PK	26° 46' N, 74°55' E
18	<i>Drimia indica</i> (Roxb.) Jessop	2n=20	Nathdwara, Rajasthan	UI-ND	24° 93' N, 73°83' E
19	<i>Drimia indica</i> (Roxb.) Jessop	2n=20	Binsar, Utranchal	UI-B	29° 24' N, 77°75' E
20	<i>Drimia indica</i> (Roxb.) Jessop	2n=20	Dehradun, Utranchal	UI-DD	30° 61' N, 77°97' E
21	<i>Drimia indica</i> (Roxb.) Jessop	2n=20	Kasargod, Kerala	UI-KG	12° 30' N, 75°03' E
22	<i>Drimia indica</i> (Roxb.) Jessop	2n=20	Shyamgaon, Maharashtra	UI-SY	16° 61' N, 73°17' E
23	<i>Drimia indica</i> (Roxb.) Jessop	2n=20	Fonda, Maharashtra	UI-F	15° 23' N, 74°39' E
24	<i>Drimia indica</i> (Roxb.) Jessop	2n=20	Sokhanpara, Maharashtra	UI-S	16° 59' N, 73°41' E
25	<i>Drimia indica</i> (Roxb.) Jessop	2n=20	Purnagarh, Maharashtra	UI-P	16° 47' N, 73°23' E
26	<i>Drimia indica</i> (Roxb.) Jessop	2n=20	Ratnagiri, Maharashtra	UI-R	17° 05' N, 73°17' E
27	<i>Drimia indica</i> (Roxb.) Jessop	2n=20	Anjuna Beach, Goa	UI-GA	15° 36' N, 73°45' E
28	<i>Drimia polyantha</i> (Blatt & McC) Ansari & Raghavan	2n=20	Shivaji University, Maharashtra	UP-SU	16° 40' N, 74°15' E
29	<i>Drimia polyantha</i> (Blatt & McC) Ansari & Raghavan	2n=20	Panhala Hill, Maharashtra	UP-PH	16° 48' N, 74°06' E
30	<i>Drimia polyantha</i> (Blatt & McC) Ansari & Raghavan	2n=20	Kas Plataue, Maharashtra	UP-K	17° 41' N, 73°49' E
31	<i>Drimia polyantha</i> (Blatt & McC) Ansari & Raghavan	2n=20	Ajara, Maharashtra	UP-AJ	16° 08' N, 73°49' E
32	<i>Drimia polyantha</i> (Blatt & McC) Ansari & Raghavan	2n=20	Halkarni, Maharashtra	UP-HL	15° 54' N, 74°17' E
33	<i>Drimia razii</i> Ansari	2n=20	Gokak, Maharashtra	UR-G	16° 10' N, 74°49' E
34	<i>Drimia razii</i> Ansari	2n=20	Divaghat, Maharashtra	UR-DG	18° 23' N, 74°01' E
35	<i>Ledebouria hyacinthina</i> Roth.	2n=30	Panhala Hills, Maharashtra	SH-PH	16° 48' N, 74°06' E
36	<i>Ledebouria hyacinthina</i> Roth.	2n=30	Halkarni, Maharashtra	SH-HL	15° 54' N, 74°17' E
37	<i>Ledebouria hyacinthina</i> Roth.	2n=30	Belgaum, Karnataka	SH-BL	14° 24' N, 75°14' E
38	<i>Ledebouria hyacinthina</i> Roth.	2n=3x=45	Kas, Maharashtra	SH-KA	17° 41' N, 73°49' E
39	<i>Ledebouria hyacinthina</i> Roth.	2n=3x=45	Ajara, Maharashtra	SH-AJ	16° 08' N, 73°49' E
40	<i>Ledebouria hyacinthina</i> Roth.	2n=4=60	Aurangabad, Maharashtra	SH-AG	19° 53' N, 75°18' E
41	<i>Ledebouria hyacinthina</i> Roth.	2n=4x=60	Divaghat, Maharashtra	SH-DG	18° 23' N, 74°01' E
42	<i>Chlorophytum viviparum</i> A. Chev.	2n=28	Delhi	C	28° 61' N, 77°17' E

1,000 replicates. PCA (Principle Coordinate Analysis) was also done on the data.

The relationship between the results obtained on the same dataset by the two different markers was analyzed using

Mantel's test for correlation between matrices (Mantel 1967). It estimates the degree of correlation between the matrices by means of a test criterion, Z , and a product-moment correlation, r .

Table 2 List of selected RAPD primers along with their sequences and some characteristics of the amplification products in Hyacinthaceae

S.No.	Primer	Sequence (5′–3′)	% polymorphism	No. of amplified products	Molecular weight range (bp)
1	E2	GGTGCGGGAA	100	13	150–1200
2	E4	GTGACATGCC	90.9	11	300–1600
3	E5	TCAGGGAGGT	100	11	1031–3000
4	E8	TCACCACGGT	100	21	300–1000
5	E10	CACCAGGTGA	92.3	13	500–(>3000)
6	E12	TTATCGCCCC	100	11	400–2000
7	E14	TGCGGCTGAG	92.8	14	300–1500
8	E17	CTACTGCCGT	92.3	13	200–1500
9	E19	ACGGCGTATG	90	10	300–1200
10	E20	AACGGTGACC	100	9	400–3000
11	OPB6	TGCTCTGCCC	78.5	14	450–2000
12	OPB8	GTCCACACGG	100	13	400–1000
13	OPB11	GTAGACCCGT	100	11	250–2000
14	OPB12	CCTTGACGCA	84.6	13	500–3000
15	OPB13	TTCCCCCGCT	100	10	200–1300
16	OPB14	TCCGCTCTGG	100	9	250–1200
17	OPA4	AATCGGGCTG	92.8	13	400–1400
18	OPA5	GGTCCCTGAC	100	16	200–1600
19	OPA7	GAAACGGGTG	90	10	500–1800
20	OPA15	AGCCAGCGAA	100	13	300–2000
21	OPA19	CAAACGTCGG	100	17	500–2000

Results and discussion

RAPD

The RAPD primers gave amplification products of different molecular weight ranging from 200 bp to 3000 bp (Fig. 2; Table 2). Total 265 amplification products with an average of 12.6 bands per primer were obtained. Minimum number of bands, nine, was obtained by primers E20 and OPB14 and maximum bands, 21, were obtained by primer E8.

High polymorphism ranging from 78.5 % to 100 % was obtained with all primers giving more than 75 % polymorphism. Eleven primers gave 100 % polymorphism and the average polymorphism present in Hyacinthaceae was 94.9 %. Among the genera, highest polymorphism was shown by *Dipcadi* (55.4 %) followed by *Drimia* (47 %) while *Ledebouria* (38.6 %) showed the least polymorphism. In genus *Drimia*, different species showed varying levels of polymorphism. *D. indica*, *D. coromandaliana*, *D. wightii*, *D. razii* and *D. polyantha* showed an average of 33.2 %, 2.13 %, 11 %, 3.17 % and 20.15 % polymorphism, respectively, among their populations. Genus specific bands for *Drimia* with primers E4, E19, E20, OPA15, for *Ledebouria* with E2, E4, OPA4, OPB6 and for *Dipcadi* with primer OPA4 were obtained, while with primer E20, 700 bp species specific band for *D. wightii* was observed.

A wide range in the values of Jaccards' similarity coefficient (0.2–0.93) was obtained among the accessions, with an average of 0.54, indicating high genetic diversity. *Dipcadi montanum* (DM-N) and *Drimia coromandaliana* (UCR-A) were the most divergent groups within the family with similarity coefficient of 0.2. Highest similarity index was observed between *D. coromandaliana* accessions (UCR-A and UCR-K) with a value of 0.931. Within *Drimia*, the least similarity

Table 3 SRAP primer pairs selected for the present study with some characteristics of the amplification products in 41 accessions belonging to three genera of family Hyacinthaceae in India

Primer pair	Number of amplified products	% Polymorphism	Molecular weight range (bp)
em1-me3	10	100	200–1200
em2-me1	12	91.6	150–1000
em2-me2	6	83.3	300–1100
em2-me3	11	100	100–900
em3-me1	13	100	400–1300
em3-me3	12	100	500–1400
em4-me1	7	100	200–1050
em4-me4	6	83.3	100–700
em4-me5	14	100	150–1150

Fig. 2 Representative band profiles of **a** RAPD marker with random decamer primers OPB 20 and **b** SRAP marker with primer combinations em2-me1, showing maximum polymorphism. M: 100 bp marker (MBI Fermentas), lanes 1–13: *Drimia indica*, 14–15: *Drimia coromandaliana*, 16–18: *Drimia wightii*, 19–20: *Drimia razii*, 21–25: *Drimia polyantha*, 26–32: *Ledebouria hyacinthina*, 33: *Dipcadi concanense*, 34: *Dipcadi serotinum*, 35: *Dipcadi saxorum*, 36–37: *Dipcadi montanum*, 38: *Dipcadi minor*, 39–40: *Dipcadi ursulae*, 41: *Dipcadi erythreum* and 42: *Chlorophytum viviparum*

coefficient (0.47) was between *D. coromandaliana* and *D. wightii*. In *Dipcadi*, least similarity coefficient (0.4) was observed between *Dipcadi concanense* and *Dipcadi erythreum*, while highest similarity (0.91) was shared between the two populations of *Dipcadi ursulae*. Within the populations of *Ledebouria hyacinthina*, the similarity coefficient ranged from 0.65 to 0.83 with highest similarity between Halkerni and Belgaum populations and lowest similarity between Aurangabad and Ajara populations. *Chlorophytum* as outgroup emerged most divergent with low similarity coefficients of 0.12–0.25.

The UPGMA based dendrogram obtained from the similarity matrix separated the 42 accessions in three clusters corresponding to the three subfamilies; Urginoideae, Ornithogaloideae and Hyacinthoideae (Fig. 3). They formed monophyletic groups supported by strong bootstrap values (96 % and 84 %). Cluster I included all species of *Drimia*, cluster II included genus *Dipcadi* and cluster III had the *Ledebouria hyacinthina* populations. Within genus *Drimia*, *Drimia wightii* emerged out as well supported (100 % bootstrap value) distinct monophyletic clade Ib. *Drimia indica* appeared to be paraphyletic group with apparently strong affinity with *Drimia polyantha*. However, *Drimia polyantha* remained a well differentiated clade within *Drimia indica*. *Drimia indica* from northern and western India grouped together while the populations from Western Ghats except population from Shyamgaon (UI-SY) clustered together. Among Western Ghat populations, triploids (UI-P, UI-S, UI-R and UI-GA) formed a weak group (27 % bootstrap value). *Drimia razii* and *Drimia coromandaliana* were separated from the *Drimia indica* clade as well differentiated taxa.

Among *Dipcadi*, all species from Western Ghats namely *Dipcadi montanum* (DM-A, DM-N), *Dipcadi minor* (DMN-H), *Dipcadi saxorum* (DSX-K) and *Dipcadi ursulae* (DU-KS, DU-T) except *Dipcadi concanense* (DC-R) clustered together while *Dipcadi concanense* separated as a very diverged species supported by strong bootstrap value (100 %). *Dipcadi erythreum* (DE-J) and *Dipcadi serotinum* (DS-D) from Jodhpur and Delhi, respectively, diverged from other species at 40 % similarity suggesting they are quite differentiated genetically. Among the Western Ghats populations, *Dipcadi ursulae* populations were distinct while *Dipcadi montanum* and *Dipcadi minor* grouped together along with *Dipcadi saxorum*.

Ledebouria hyacinthina populations were found to be quite differentiated from each other with population from Aurangabad (SH-AR) to be the most divergent with 100 % bootstrap value. Populations from Divaghat (SH-D) and Kas (SH-KS) were also found to be genetically distinct while the rest of the populations formed a loose clustering.

In PCA analysis, first component accounted for 27 % of the total and the second and third component accounted for 15 % and 8 % respectively. The three genera distinctly clustered as three different groups in the PCA biplot. However, 2n and 3n populations of *D. indica* and 2n, 3n and 4n populations of *Ledebouria hyacinthina* could not be differentiated from each other.

SRAP

Nine primer combinations giving consistent, scorable and reproducible amplification pattern were selected for studying

Fig. 3 Rooted UPGMA dendrogram based on Jaccard similarity matrix showing the relationship among members of family Hyacinthaceae in India on the basis of combined data of RAPD and SRAP markers

the 41 accessions (Fig. 2). A total of 91 amplification products were obtained ranging in size from 150 bp to 1,400 bp, out of which, 88 were polymorphic. Number of bands per primer pair varied from 6 (em2-me2 and em4-me4) to 14 (em4-me5) with an average of 10.1 bands per primer pair. Extent of polymorphism obtained was high ranging from 83.3 %–100 % with six primers giving 100 % polymorphism and average polymorphism of 96.7 % in the family Hyacinthaceae (Table 3).

Genus specific bands for *Dipcadi* with primers em1-me3 (910 bp) and em4-me4 (200 bp) and for *Ledebouria* with primer pairs em3-me3 (500 bp) and em4-me4 (350 bp) were observed. Species-specific band for *Drimia wightii* was obtained with em1-me3 (1,150 bp). Among the genera, *Dipcadi* showed the highest polymorphism (70.5 %) followed by *Ledebouria* (56.7 %) and *Drimia* (48.9 %). Within the genus *Drimia*, the species showed low average polymorphism in *D. indica* (17.8 %), *D. coromandaliana* (2.7 %), *D. wightii* (2.9 %), *D. razii* (9.4 %) and *D. polyantha* (14.9 %).

Pair-wise Jaccard similarity coefficient showed a wide range of values from 0.06 to 1 with an average value of 0.46. The most divergent taxa observed were *Dipcadi ursulae* and *Drimia indica* with lowest similarity coefficient of 0.06.

Two populations of *Drimia indica* from Jodhpur and Delhi (UI-J and UI-DL) and two populations of *Drimia polyantha* from Ajara and Halkarni (UP-A and UP-HL) had similarity coefficient of 1 and the markers could not distinguish these populations from each other. Within the genera, the values of similarity coefficient ranged from 0.36 to 0.88 in *Dipcadi*, 0.17 to 0.8 in *Ledebouria* and 0.55 to 1 in *Drimia*. Within *Drimia*, *Drimia wightii* and *Drimia indica* showed least similarity with each other while in genus *Dipcadi*, *Dipcadi erythreum* and *Dipcadi concanense* came out to be most diverged species.

In UPGMA dendrograms as well as neighbor joining trees, three major clusters were apparent. Cluster I and II comprised of the genera *Drimia* and *Ledebouria* while cluster III included all the species of *Dipcadi*. *Drimia wightii* separated as a well distinct and diverged taxon supported by high bootstrap value (99 %). All the populations of *Drimia indica* grouped together into a well distinct clade, however, the populations did not grouped according to their geographical ranges and occurred intermixed in the cluster. In SRAP marker dendrogram, *Drimia polyantha* grouped with *Drimia coromandaliana* and *Drimia razii*. Populations of *Drimia*

polyantha from Kolhapur and Panhala hills occurred closer to *Drimia coromandaliana* while the other three populations were clustered together. *Drimia coromandaliana* also came out to be a well differentiated taxon in the tree.

Populations of *Ledebouria hyacinthina*, seemed to be quite diverged and differentiated from each other. However, in the present study, polyploids of *Drimia* and *Ledebouria* could not be differentiated from their respective diploid populations in the clustering pattern.

In genus *Dipcadi*, *Dipcadi concanense* emerged as the most diverged from the other *Dipcadi* species with branching at 47 % similarity and *Dipcadi erythreum* from Jodhpur and *Dipcadi serotinum* from Delhi were well differentiated taxa in neighbor joining tree. While in UPGMA dendrogram, *Dipcadi concanense* grouped with *Dipcadi serotinum* whereas *Dipcadi erythreum* clustered along with the other Western Ghats species.

All the species from Western Ghats except *Dipcadi concanense* (*D. saxorum*, *D. montanum*, *D. minor* and *D. ursulae*) grouped together. Among the Western Ghat species, *Dipcadi ursulae* was diverged from *Dipcadi saxorum*, *Dipcadi montanum* and *Dipcadi minor* as the latter three species clubbed together. *Dipcadi minor* considered being a narrow leaved form of *Dipcadi montanum* grouped together.

In PCA analysis, the three genera were very well differentiated and distinct. Species of *Dipcadi* were more widely placed than the *Drimia* populations suggesting the former genus to be more heterogeneous genetically while the latter to be genetically more homogenous. However, *Ledebouria hyacinthina* populations showed divergence higher than the species of *Drimia*. Thus, *Ledebouria hyacinthina* appears to be genetically diverse comparable to the diversity present in genus *Dipcadi*. The first component in the PCA accounted for 36.4 % variation and the second and third component accounted for 15.2 % and 8.9 % variation, respectively.

Based on the RAPD and SRAP studies, the taxa were clearly resolved into three groups corresponding to three sub-families; Urginoideae, Hyacinthoideae and Ornithogaloideae (Pfosser and Speta 1999) of Hyacinthaceae. Within the genus, *Drimia wightii* appeared to be the most distinct and divergent from the remaining species suggesting the species to be genetically differentiated from other *Drimia* spp. With both the marker systems, species specific markers for *D. wightii* were obtained. This observation is in accordance with the earlier studies based on hybridization (Dixit and Yadav 1989) showing that *D. wightii* did not cross with any other *Drimia* sp. The species is also morphologically distinct from other species with respect to leaf size, shape, colour and most importantly inflorescence axis which is very condensed in *D. wightii* while it is long in case of all other *Drimia* spp. *Drimia indica* and *D. polyantha* populations could not be distinguished with RAPD and combined analysis (RAPD and SRAP) implying that *D. indica* is closer to *D. polyantha*. In hybridization studies

reported earlier *Drimia indica*, *Drimia polyantha* and *Drimia coromandaliana* showed close relationship and formed a species complex (Dixit and Yadav 1989). The five species of *Drimia* under investigation can be divided into day blooming (*D. wightii*, *D. razii* and *D. polyantha*) and night blooming (*D. indica* and *D. coromandaliana*). Pollen structure also supports this grouping with large pollen grains with coarse reticulate ornamentation in night blooming group and small with fine reticulate ornamentation in day blooming group. However, interestingly, *Drimia polyantha* is reported to show intermediate conditions between the two groups (Dixit and Yadav 1989). Thus, the peculiar placement of *Drimia polyantha* in our results and the earlier reports suggests need to reassess the distinct species status of *Drimia polyantha*.

The species of *Dipcadi* belonging to subfamily Ornithogaloideae appears a monophyletic group. The taxonomy of the genus *Dipcadi* is poorly defined because of the taxonomically bad morphological characters (Dixit et al. 1992). Uniform and overlapping morphology of different species makes the genus taxonomically difficult at species level. Moreover, the characters show great plasticity with the environmental conditions. However, *Dipcadi concanense* has emerged out to be a very distinct species from the other species of Western Ghats namely *D. montanum*, *D. minor*, *D. saxorum* and *D. ursulae* which grouped together. It is supported by the morphology as *Dipcadi concanense* is very distinct from the other *Dipcadi* spp. with glistening white very long (2.5–4.5 cm) flowers as against the greenish-brownish-grey-white 0.8–1.7 cm long flowers in other species. Among the other Western ghat species, *Dipcadi ursulae* populations were distinct while *Dipcadi montanum* and *Dipcadi minor*, latter considered being a narrow leaved form of the former, grouped together along with *Dipcadi saxorum*. The Western Ghat species with an exception of *Dipcadi concanense* were found to be genetically close forming a well distinct group, whereas, northern Indian species, *Dipcadi erythreum* from Rajasthan and *Dipcadi serotinum* from Delhi stood out as well differentiated taxa. Interestingly, *Dipcadi serotinum* flowers in February–March against the other *Dipcadi* species in India that flower in June–July suggesting it to be an introduction from Europe where *Dipcadi serotinum* is widely distributed. *Dipcadi erythreum* is also coming out as a genetically distinct species and is restricted to hilly desert tracts of Rajasthan indicating that it might be an introduction from Pakistan and Baluchistan. It is suggested that Indian *Dipcadi* consist of three different stocks viz. *D. serotinum*, a temperate stock from Europe, *D. erythreum* from Pakistan (possibly from Mediterranean) and the remaining species constituting the Indian stock restricted to Western Ghats.

All *Ledebouria hyacinthiana* populations were grouped together. Only one species of *Ledebouria* is included in the study because the other two species, *L. hoehankeri* and *L. viridis* have been collected only once from their type localities

and have not been collected again by anyone. *Ledebouria* was earlier included in the genus *Scilla* but Indian species used in the present study were observed to have the characters scored for *Ledebouria* by Jessop (1970) like presence of spotted leaves in some populations, presence of 1–4 flexuose inflorescence per plant with purple-pink flowers and one ovule per locule. Seven populations of *Ledebouria hyacinthina* though collected from west coast of India, showed high genetic divergence having relatively lower pairwise similarity coefficient range than the genus *Dipcadi*. They seem to diverge at the similarity level where the different species of *Drimia* and *Dipcadi* have diverged. Moreover, the populations are found to be morphologically quite distinct with an astonishing level of diversity in leaf size, colour, shape, presence or absence of black spots on the leaves, ploidy levels and their habitat preferences. Therefore, the high genetic divergence obtained among the populations is in agreement to the morphological diversity present. Earlier, Van Staden and Pan (2001) employed RAPD in three species of genus *Scilla* from Africa and concluded that RAPD can be applied successfully for the identification of different species of *Scilla*. In the present study, different populations of the species could be differentiated very distinctly by RAPD and SRAP markers.

AMOVA among the three genera revealed that 66.8 % of the total variability is accounted among genera while only 20.3 % and 12.8 % of the variability is accounted among species within genera and within species respectively (Fig. 4).

On estimation of partitioning of variance between widely distributed *Drimia indica* and other species of *Drimia* having restricted distribution in Western Ghats, 51.5 % of the variance was found to be among populations within species while 26.6 % and 21.7 % of the variability was within populations and among species respectively (Fig. 4).

The three genera came out to be quite diverged and well differentiated in AMOVA analysis as most of the variance was observed among the genera. Negative variance was observed between the widespread *Drimia indica* and other species of *Drimia* with restricted distribution which implies that loci analyzed are more related between than within populations (Weir 1990). The biological cause of negative component of variance is in the light of avoiding self mating that allows the genes to be more alike between individuals than within individual.

Among the three genera, *Dipcadi* showed highest polymorphism suggesting it to be more diverse genus than *Drimia* inspite of more widespread distribution of *D. indica* throughout the country in different climatic conditions. Similar trends of restricted species having higher levels of genetic variations than the widespread species has been reported (Nickerent and Weins 1990). *Dipcadi* seem to have a broader genetic base than *Drimia* that may be attributed to genetic bottleneck in the founder germplasm of the genus *Drimia* in India. Moreover,

Fig. 4 Schematic representation showing partitioning of total variance into three components based on SRAP. The outermost ring denotes the partitioning of variance among the three genera viz. *Drimia*, *Dipcadi* and *Ledebouria*, middle ring denotes the variance components in *Drimia indica* and other *Drimia* spp. And the innermost ring represents variance components in *Drimia indica* and *Drimia coromandaliana*

Dipcadi in India appears to be introduced from different genetic stock including European and Mediterranean regions. The basic chromosome numbers in the species of *Dipcadi* are variable while in case of *Drimia* all the species have the same basic chromosome number. Thus, it may be concluded that speciation in *Dipcadi* is more diversified undergoing changes in basic chromosome number while in *Drimia*, speciation has accompanied only structural rearrangements in the genome. Low polymorphism in *Ledebouria* might be because only one species of *Ledebouria*, though different cytotypes, were studied.

The family Hyacinthaceae in India seem to be very diverse with high polymorphism obtained with the marker systems studied. Within *Drimia*, *D. indica* showed highest polymorphism. The populations were collected from different parts of the country and the collection sites are separated by large geographical distances. Geographical distances of *D. polyantha* populations are also considerable resulting in relatively high interpopulation polymorphism while collection site of *D. razii*, *D. coromandaliana* and *D. wightii* populations were closer resulting in low polymorphism. Thus, geographical distances correlated well with the level of polymorphism in congruence with Wrights' neighbourhood model (isolation by distance) (Wright 1946). In case where gene flow between neighbouring populations is not limited, population of longer geographical distances generally shows greater diversification. Similar kind of results were obtained in another study where closer the geographical locations of the populations, the closer genetic relationships between them were observed in *Lilium longiflorum* belonging to the Liliaceae, a related and erstwhile family of the genus *Drimia* (Wen and Hsiao 1999).

Endemic species namely, *D. razii*, *D. wightii* and *D. coromandaliana*, in general, showed lower polymorphism than widely distributed *D. indica*. Endemic and rare species, as a consequence of genetic drift in small populations and strong directional selection operating in a limited array of environments, tend to have genetic uniformity or decreased levels of genetic variation (Wright 1931; Dodd and Helermun 2002). On the other hand, due to diversified selective forces across the wide range of environmental conditions and isolation of populations, the widespread species generally show higher levels of genetic diversity (Baskauf et al. 1994).

Combined data of RAPD and SRAP

Mantel matrix correspondence test was used to test the correlation between the clustering pattern and similarity coefficients obtained with RAPD, SRAP and combined data. The correlation coefficients obtained were statistically significant. A high correlation ($r=0.932$) was obtained between the matrices obtained by RAPD and SRAP markers on the 13 species belonging to 3 genera of Hyacinthaceae in India (Table 4). The combined data of the two markers also gave a very good correlation with the respective markers individually. The clustering pattern based on combined data was more similar to RAPD data (Fig. 3). Thus, RAPD analysis provided an efficient tool in addressing the genetic diversity and relationship among the three genera. *Drimia*, *Dipcadi* and *Ledebouria* emerged out to be well-differentiated and distinct groups. *Drimia wightii* stands out a quite divergent species of the genus and *Drimia coromandaliana* appeared to be of a distinct species rather than a mere polyploid of *Drimia indica*. In addition, polymorphism among the populations of the same species is in direct correlation with the geographical distances between them. The grouping of taxa on basis of SRAP confirmed the RAPD based grouping as well as morphological based delimitation of species. High correlation ($r>0.9$) was obtained between RAPD and SRAP markers which indicate the very good fit correlation between RAPD and SRAP based similarities. RAPD resulted in more numbers of markers per primer (12.1) than SRAP, however, SRAP was found to be more polymorphic (96.7 %) than the RAPD (94.9 %). Similar reports of higher polymorphism in SRAP than RAPD has been there in *Dactylis glomerata* (Zeng et al. 2008). Budak

et al. (2004) used SRAP, ISSR, RAPD and SSR for genetic relationship and phylogenetic analysis of buffalograss. They analyzed both seeded and vegetative biotypes and found SRAP markers to have high discriminating power as compared to others in order SRAP>SSR>ISSR>RAPD.

The trees based on these multilocus markers viz. RAPD and SRAP were found to be more in agreement with the species tree i.e. morphology based than the unilocus nuclear as well as organellar markers (data not shown here). Despres et al. (2003) also reported that use of cpDNA (*trnL* intron and *trnL-F* spacer) and ITS region was not informative in inferring phylogeny in closely related species of genus *Trollius* (Ranunculaceae) while AFLP markers proved to be powerful tools in revealing the complex genetic relationships through robust cladistics and phonetic trees. Similarly, Potokina et al. (1999) reported RAPD to be more informative in inferring relationships among closely related taxa as greater number of polymorphic sites throughout the whole genome could be analyzed. Freisen et al. (1999) also concluded that although cpDNA loci could distinguish *Allium fistulosum* and *A. altaicum*, RAPD could clarify the interrelationship of the two taxa. The gene trees based on cpDNA and ITS might not represent the true species tree either due to lack of enough nucleotide variability at the desired taxonomic level or random sorting of polymorphic alleles in different lineages. Therefore, use of many sequences through the use of multilocus markers such as AFLP, RAPD or SRAP covering the larger part of the genome can be more useful for yielding better resolution in closely related taxa than the unilocus cpDNA and ITS sequences.

Conclusions

Our studies based on the markers used support the inclusion of three genera belonging to family Hyacinthaceae occurring in India in three distinct subfamilies, namely, Urginoideae, Omithogaloideae and Hyacinthoideae because of their separation in three clusters without exception. Thus, the three genera viz. *Drimia*, *Dipcadi* and *Ledebouria* are monophyletic groups.

Within genus *Drimia*, *Drimia wightii* is the most distinct species among the five species. *Drimia razii* is also a well diverged species and always groups as a monophyletic clade. *Drimia coromandaliana* appears to be a well differentiated species rather than being an allotetraploid of *Drimia indica*. *Drimia polyantha* seems to be close to *Drimia indica* in RAPD based tree as it clusters within *Drimia indica*. Thus, *Drimia indica* seems to be paraphyletic group.

Within the genus *Dipcadi*, species of the Western Ghats namely, *Dipcadi ursulae*, *D. minor*, *D. montanum* and *D. saxorum* appear to be closely related. However, among the Western Ghats species, *Dipcadi concanense* is very distinct

Table 4 Cophenetic correlation coefficient comparison of similarity matrices and cluster pattern from dataset obtained using RAPD, SRAP and combined data using Mantel test

Marker system	RAPD	SRAP	Combined data set
RAPD	0.987	0.932	0.993
SRAP		0.987	0.966
Combined data set			–

species and is always grouped away from the other species of the area. *Dipcadi erythreum* and *Dipcadi serotinum* are much diverged from the species of Western Ghats. The populations of *Ledebouria hyacinthina* appears very much diverged from each other and it can be suggested that *Ledebouria hyacinthina* is a species complex. Stedje (1998) has similarly suggested species complex *Ledebouria revoluta* (Syn. *Ledebouria hyacinthina*) with populations of this species from different areas showing divergence and clustering as paraphyletic group. In our results also the populations of *Ledebouria hyacinthina* from different areas and ploidy levels showed relatively high within-group divergence and appeared paraphyletic group. Thus, *Ledebouria hyacinthina* shows a significant line of cytogenetic evolution and diversification (Dixit et al. 1989). As Stedje (1998) has concluded, the species might be containing several biological species even if no morphological discontinuities seem to exist. However, further studies aimed at studying the extent of crossability among these populations are required to finally assign it as a 'species complex'.

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