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Assessment of genetic variation among populations of *Rhynchostylis retusa*, an epiphytic orchid from Goa, India using ISSR and RAPD markers

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Rhynchostylis retusa (L.) Bl., a monopodial epiphytic orchid species with attractive flowers arranged in racemose inflorescence, ranks among the important Indian ornamental orchids. Comparative population studies using PCR based markers, RAPD and ISSR, were performed to assess the genetic diversity of the wild orchid. Among the 35 primers tested, 13 RAPD and 7 ISSR primers were selected for the analysis. In total, 74 RAPD and 30 ISSR fragments were generated. High level of polymorphism was recorded in RAPD (76.13%) than ISSR (62.6%). In case of RAPD, Nei's average genetic identities value for different populations of *R. retusa* ranged from 0.405 to 0.932. While for ISSR, it ranged from 0.733 to 0.933. The results of the present study can be seen as starting point for future research on the population and evolutionary genetics of this species.

Keywords: *Rhynchostylis retusa*, RAPD, ISSR, genetic variation

Introduction

Orchidaceae is one of the largest families of flowering plants[1] and orchids are marketed as both plant and cut flower. The largest exporters of potted orchids are Taiwan, Thailand, the United Kingdom, Italy, Japan, New Zealand and Brazil, while the largest importer of potted orchids is the United States[2]. Orchids are currently the second most valuable potted crop in the United States with a total wholesale value of $144 million[3]. In India, however, floriculture is picking up as an industry with a turn over of about, $ 47 million[4].

Rhynchostylis retusa (L.) Bl. is a monopodial, epiphytic orchid species with beautiful flowers arranged in racemose inflorescence, which rank the species among the important Indian ornamental orchids (Fig. 1). Despite the tremendous diversity within the family[5], very little is known about the genetic diversity within natural populations. Habitat destruction, macroclimatic changes and shifting cultivation deplete orchid populations[6]. Many natural populations have been destroyed or restricted to smaller size for habitat destruction and fragmentation caused by various developmental activities. Deleterious effect of habitat destruction and over exploitation of wild orchids has been reported from different part of world[7-9]. Comparative population studies using DNA based marker systems are emphasized to collect information on the level and pattern of genetic diversity of wild orchids, which is the first step to facilitate their conservation. Knowledge about genetic diversity is considered the baseline for conservation[10].

To study the genetic variation in various plant species, a number of DNA based reliable marker systems have been developed. These markers have been used widely, either alone or in tandem with morphological markers, to obtain more consistent information on the existing genetic diversity with a number of species groups. The techniques such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), inter simple sequence repeats (ISSR) and simple sequence repeats (SSR) are used frequently to study plant diversity. RAPD and ISSR are two simple and quick techniques, where the former detects nucleotide sequence polymorphism using a single primer of arbitrary nucleotide sequence[11] and the latter permits detection of polymorphism in inter-microsatellite loci using a primer designed from dinucleotide or trinucleotide simple sequence repeats[12-13].

The present study is aimed to explore the extent of genetic variation among the wild populations of *R.*

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retusa, which would help in better understanding of genetic profile that can be used to develop strategies for its conservation and sustainable utilization.

Materials and Methods

Plant Material

Leaf samples of *R. retusa* representing seven different natural populations in Goa were collected. Leaves were stored at 4°C in zip-lock plastic bags till they were processed for DNA isolation. The locations from where the leaf samples are collected are illustrated in Fig. 2. List of populations, their altitude and other details are given in the Table 1.

Fig. 1—*R. retusa* in flowering

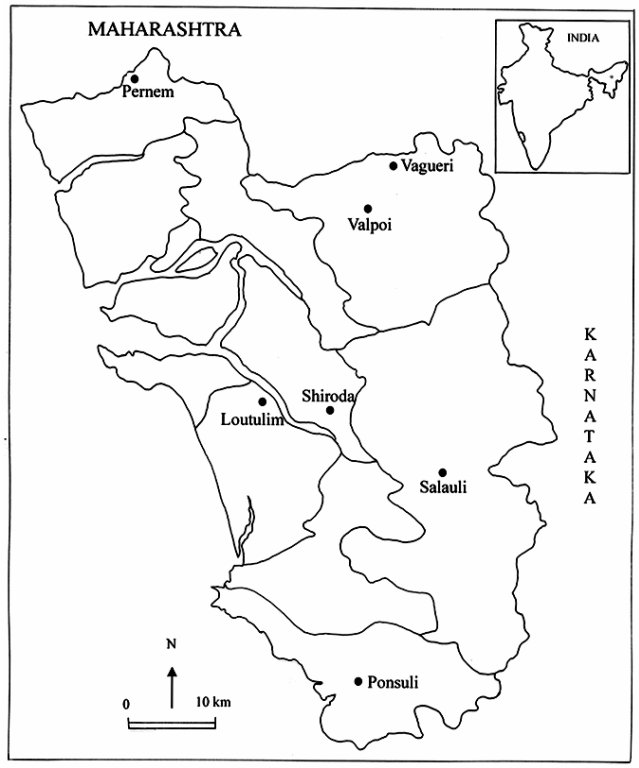

Fig. 2—Map of Goa showing collection site of different populations

Genomic DNA Extraction

DNA was isolated using a modified CTAB (cetyl trimethyl ammonium bromide) method[14]. For each accession, about 5 g of bulked leaf tissue from 50 plants was ground to a fine powder using liquid nitrogen, which was then suspended in 20 mL of extraction buffer (20 m*M* EDTA at *p*H 8.0, 100 m*M* Tris-HCl at *p*H 8.0, 1.5 *M* NaCl, 2% CTAB and 1% β-mercaptoethanol). The suspension was mixed well, incubated at 60ºC for 45 min, followed by chloroform:isoamyl alcohol (24:1) extraction and precipitation with 0.6 volume of isopropanol at –20ºC for 1 h. The DNA was pelleted down by centrifugation at 12,000 rpm for 10 min and was then suspended in TE buffer (10 m*M* Tris-HCl and 1 m*M* EDTA, *p*H 8.0). The DNA was purified from RNA and proteins by standard procedures[15], and its concentration was estimated by agarose gel electrophoresis and staining with ethidium bromide.

RAPD Amplification

Twenty RAPD primers obtained from Operon Technologies, USA were tested initially with randomly selected individuals from two populations. Thirteen primers that showed clear and reproducible results were chosen for the study. PCR amplification was performed in a 25 µL-reaction volume containing 50 ng of genomic DNA, 2.5 µL of 10-X Taq assay buffer (100 m*M* Tris-HCl, *p*H 8.3; 20 m*M* $MgCl_2$; 500 m*M* KCl; 0.1 % gelatin), 2.5 µL of 2.5 m*M* dNTPs, 15 ng of primers and 0.5 units of Taq polymerase (Bangalore Genei).

The mixture was subjected to PCR on Perkin-Elmer 480 thermal cycler programmed for an initial step of 3 min at 94°C, followed by 40 cycles of 94°C

Table 1—Collection sites of *R. retusa* populations

Populations	Latitude	Longitude	Altitude (m)
Ponsuli	14° 59′ 56.9”N	74° 02′ 34.6” E	10
Pernem	15° 43′ 31.6”N	73° 47′ 47.2” E	15
Salauli	15° 09′ 28.01”N	74° 13′ 47.7” E	76
Valpoi	15° 33′ 26.01” N	74° 04′ 36.01”. E	71
Shiroda	15° 27′ 31.01” N	73° 59′ 55.6”E	102
Loutulim	15° 23′ 06.6” N	73° 56′ 22.5” E	103
Vagueri	15° 34′ 59.8” N	74° 05′ 47.6” E	295

for 1 min, 37°C for 1 min and 72°C for 2 min and 10 min for final extension step at 72°C. The amplification products were analysed on 1.4% agarose gels with mol wt marker (1 Kb Marker, GeneRuler™, MBI Fermentas, USA). The gel was stained with ethidium bromide, visualized under ultraviolet light and photographed using gel documentation system (Amersham Pharmcia Biotech VDS Image master).

ISSR Amplification

Fifteen ISSR primers from Operon Technologies, California, USA was initially tested. Out of 15 primers, 7 showed clear and reproducible bands and they were used during the study. The amplification was performed in a 25 µL reaction volume containing 5 ng of genomic DNA, 2.5 µL of 2.5 m*M* dNTP's, 25 pmol/µL of primer and 0.6 units of Taq polymerase. Initial denaturation was for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, annealing temperatures varies for different primers (Table 2) and 2 min at 72°C. An additional cycle of 10 min at 72°C was used for primer extension. The PCR products were analysed on 1.2% agarose gels and stained with ethidium bromide, photographed under ultraviolet light by using gel documentation system. Mol wt was estimated by using 1 Kb DNA ladder (GeneRuler™).

Data Analysis

ISSR and RAPD amplified fragments named by primer code and mol wt (bp) were scored as band present (1) or absent (0) for each DNA sample. Dendrogram and genetic distance was generated by using (simple matching coefficient) the POPGENE program[16].

Results

Among the total primers tested, 13 RAPD and 7 ISSR primers that showed clear and reproducible banding pattern are given in Fig. 3 and Tables 3 and 4.

Table 2—List of ISSR primers and their annealing temperatures

No.	ISSR Primers	Annealing temperatures
1	HB 10, HB12, HB13, HB 14, HB15	39° C for 30 sec
2	17898A, 17898B, 17899A, 17899B	44° C for 30 sec
3	HB 8, HB 9	46° C for 30 sec
4	814, 814 A	53° C for 1 min
5	844 B, P2	57° C for 30 sec

RAPD Banding Pattern

The thirteen primers were chosen to generate 74 RAPD fragments, an average of 5.69 bands per primers, of which 57 bands are polymorphic for all the populations. The percentage polymorphism across all the samples varied from 33.3 to 100% (average 76.13%). The polymorphism was the highest in OPA-12, 14 and OPD 11 primers, while it was the lowest in

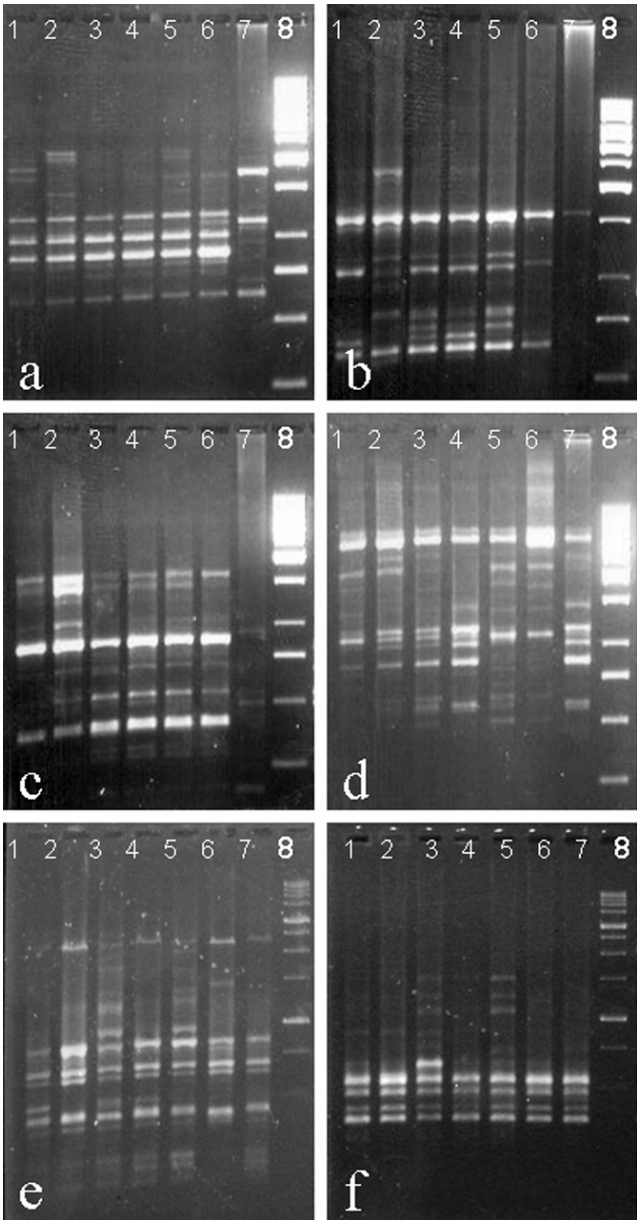

Fig. 3—RAPD and ISSR amplification profile of *R. retusa* with different primers. RAPD primers (a-d): a. Primer OPA 13, b. OPD 2, c. OPA 9, d. OPD 8; Lines (1-8): 1. Pernem, 2. Ponsuli, 3. Salauli, 4. Valpoi, 5. Shiroda, 6. Loutulim, 7. Vagueri, 8. Gene ruler™ 1 Kb DNA ladder. ISSR primers (e-f.): e. HB 12, f. HB 14; Lines (1-8): 1. Pernem. 2, Ponsuli. 3, Valpoi. 4, Loutulim. 5, Shiroda. 6, Salauli. 7, Vagueri. 8. Gene ruler™ 1 Kb DNA ladder.

Table 3—Primer sequences, amplified bands, polymorphic bands and percentage polymorphism in RAPD analysis.

No.	Primer	Sequence 5′ to 3′	Amplified bands	Polymorphic bands	% polymorphism
1	OPA 03	AGTCAGCCAC	7	5	71.4
2	OPA 04	AATCGGGCTG	6	4	66.6
3	OPA 08	GTGACGTAGG	3	1	33.3
4	OPA 09	GGGTAACGCC	7	5	71.0
5	OPA 12	TCGGCGATAG	4	4	100
6	OPA 13	CAGCACCCAC	6	5	83.3
7	OPA 14	TCTGTGCTGG	4	4	100
8.	OPD 02	GGACCCAACC	8	7	87.5
9.	OPD 03	GTCGCCGTCA	5	4	80.0
10.	OPD 05	TGAGCGGACA	6	2	33.3
11.	OPD 07	TTGGCACGGG	5	4	80.0
12.	OPD 08	GTGTGCCCCA	6	5	83.3
13.	OPD 11	AGCGCCATTG	7	7	100
Total			74	57	
Mean			5.69	4.38	76.13

Table 4—Primer sequences, amplified bands, polymorphic bands and percentage polymorphism in ISSR analysis

S.No.	Primer	Sequence 5′ to 3′	Amplified bands	Polymorphic band	% polymorphism
1	17899A	CACACACACACAAC	5	2	40.0
2	17899B	CACACACACACAGG	3	2	66.6
3	HB 9	GTGTGTGTGTGTGG	10	6	60.0
4	HB 12	CACCACCACGC	3	2	66.6
5	HB 13	GAGGAGGAGGC	5	4	80.0
6	17898 B	CACACACACACAGT	2	-	-
7	HB 15	GTGGTGGTGGC	2	-	-
Total			30	16	
Mean			4.28	3.2	62.6

Table 5—Genetic identity of *Rhynchostylis retusa* populations based on RAPD primer amplification

Ponsuli	Ponsuli	Pernem	Salauli	Valpoi	Shiroda	Loutulim	Vagueri
Pernem	-	-					
Salauli	0.702	0.851	-				
Valpoi	0.770	0.770	0.918	-			
Shiroda	0.743	0.797	0.864	0.864	-		
Loutulim	0.729	0.837	0.851	0.824	0.932	-	
Vaugeri	0.473	0.500	0.486	0.432	0.405	0.445	-

OPD-5 and OPA-8 primers. Nei's average genetic identity value ranged from 0.405 to 0.932 with the average of 0.709 for different population compared (Table 5). The dendrogram (Fig. 4) was constructed based on simple matching coefficients taking into account the presence or absence of bands. These showed four populations forming two clusters. The first cluster comprised of two populations from Salauli and Valpoi at similarity of 90%. The second clusters at similarity of 94% consists of populations from Shiroda and Loutulim. However, the other three populations Pernem, Ponsuli and Vagueri formed operational taxonomic units.

ISSR Banding Pattern

The seven ISSR primers tested, generated total 30 RAPD fragments, an average of 4.28 bands per primer. Sixteen bands, out of 30 bands, were polymorphic with the mean of 3.2 bands per primer (Table 4). The percentage of polymorphism across all the samples varies from 40 to 80% (average 62.6%). The polymorphism was the highest in primer HB 13, while it was the lowest in primer 17899A. The ISSR derived data were used to calculate the genetic identities (Table 6). The genetic similarity co-efficient varied from 0.733 to 0.933 with the average of 0.844. Simple matching coefficient method was used to

Table 6—Genetic identity of *R. retusa* populations based on ISSR primer amplification

Ponsuli	Ponsuli	Pernem	Salaulim	Sattari	Shiroda	Loutulim	Vagueri
Pernem	0.800	-					
Salauli	0.866	0.800	-				
Valpoi	0.933	0.800	0.866	-			
Shiroda	0.900	0.833	0.900	0.833	-		
Loutulim	0.866	0.866	0.733	0.800	0.833	-	
Vagueri	0.833	0.900	0.766	0.900	0.800	0.900	-

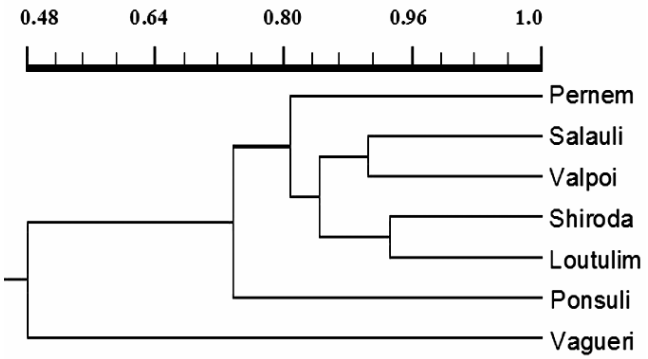

Fig. 4—Dendrogram of Nei's genetic identities between the populations of *R. retusa* based on RAPD data

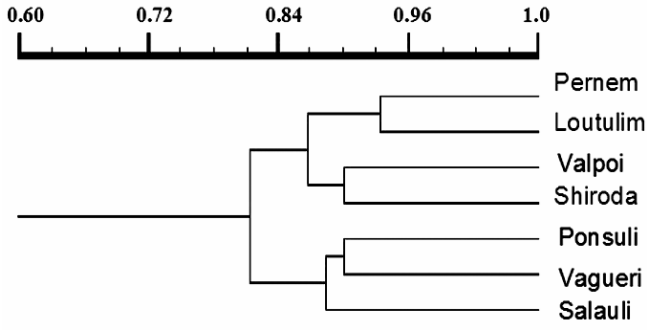

Fig. 5—Dendrogram of Nei's genetic identities between the populations of *R. retusa* based on ISSR data

construct dendrogram (Fig. 5). The populations under study were grouped into 3 groups. The first cluster formed between Pernem and Loutulim populations at 94% similarity. The populations from Valpoi and Shiroda formed the second cluster at 90% similarity. While the populations of Ponsuli, Vagueri and Salauli formed the third cluster.

Discussion

Population genetics of large number of plant species were studied using RAPD[17-19]. The level of genetic diversity of populations as well as the degree of gene differentiation between the populations is important for genetic conservation[20]. The maintenance of genetic diversity is considered crucial for long-term survival and the evolutionary response of population to adapt to the changes in the environment[21-22].

In the present study, the applicability of ISSR and RAPD was compared as genetic marker to characterize the population of *R. retusa*. The results indicate that the percentage of RAPD polymorphic bands (76.13%) was higher than that of ISSR analysis (62.6%). The mean number of amplified RAPD bands was 5.69 bands per primer, which was more than that of ISSR (4.28). The results also suggest that RAPD markers were superior to ISSR markers in the capacity of revealing more polymorphic bands. Similar results were obtained for *Citrus* sp. which showed RAPD PCR had a higher level of variations than ISSR[23]; whereas these two markers produced similar level of polymorphism in *Phaseolus vulgaris*[24].

In the present study, it is obvious that the dendrogram based on RAPD marker (Fig. 4) is not in accord with the dendrogram based on ISSR marker (Fig. 5). The dendrogram generated by RAPD matrix agrees better with the altitude of the populations (Fig. 4). The altitudes of the sample collection sites were widely varying for population to population and it ranges from 10 to 295 m (Table 1). Interestingly, almost similar altitude populations from Salauli (76 m) and Valpoi (71 m) formed one cluster, while Shiroda (102 m) and Loutulim (103 m) formed another. The populations from Pernem (15 m) and Ponsuli (10 m) formed out separate branches from the major two clusters. In addition, population from Vagueri (295 m) had a totally separate branch from all other populations since the altitude of this population is much higher in comparison with all other populations. Relatively low genetic identity of Vagueri population with other populations signifies its isolation due to higher altitude (Table 1). Genetically distinct isolated populations were also reported in *Laelia speciosa*, an endangered orchid from Mexico, using isozyme studies[25]. The distinctiveness of Vagueri population may represent

unique evolutionary process. Similar observation was reported in another orchid species, *Paphiopedilum micranthum* from South-western China[26].

Highest genetic distance was observed between populations of Vagueri and Shiroda. These two populations come from different regions indicating that geographical isolation is one of the important factors for the observed genetic distance. Shiroda and Loutulim populations have the closest genetic identities as they are morphologically, ecologically and geographically close to each other (Fig. 2). While Salauli and Valpoi populations coming under Western Ghats belt region of Goa state share the next highest genetic identities. Ponsuli and Pernem populations were close to each other as they are confined to coastal area and also at low altitude level (Table 1).

Dendrogram generated by ISSR matrix agree with the geographical location of the populations. Pernem and Loutulim populations share the highest genetic similarity, as they are in plains. Populations close to the hilly areas, i.e. Valpoi and Shiroda, shared 90% similarity. Ponsuli, Salauli and Vagueri populations formed a separate cluster. Salauli and Vaugeri were grouped together since they are almost in the parts of Western Ghats of Goa region, while closeness of Ponsuli population could not be explained. Combined ISSR and RAPD data of *Aerides maculosum*, another epiphytic orchid from the same localities as that of *R. retusa*, clearly separated the populations of hilly and forest areas from the remaining areas. It shows that pattern of gene flow is more or less identical as both are epiphytic and are from the same localities[19].

The present study reveals that within the small region taken up for study, higher genetic variability exists among the populations of *R. retusa*.

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