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Full Length Research Paper

Genetic diversity in *Oroxylum indicum* (L.) Vent. (Bignoniaceae), a vulnerable medicinal plant by random amplified polymorphic DNA marker

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Random amplified polymorphic DNA (RAPD) markers were used to assess genetic diversity in *Oroxylum indicum* (L.) Vent (Bignoniaceae) a vulnerable medicinal plant collected from eight locations in Andhra Pradesh, India. High level of genetic similarity was observed in the collected accessions. Forty random primers, each with 10 bases generated a total of 188 polymorphic bands out of the 387 total bands, that is, polymorphism of 49.61% was observed. Overall genetic similarity based on 40 random primers was 87%. Cluster analysis based on Dice coefficient showed two major groups. The results show that the genetic diversity of this species is low, possibly depicting a difficulty in adapting to environmental variations. This distributive pattern of genetic variation of *O. indicum* accessions provides important baseline data for conservation and collection strategies for this species. The collected accessions were introduced to University of Hyderabad field gene bank along with other redlisted plants of Deccan ecoregion.

Keywords: Conservation, genetic diversity, *Oroxylum indicum*, RAPD (Random Amplified Polymorphic DNA).

INTRODUCTION

Oroxylum indicum (L.) Vent. monotypic genus, (Bignoniaceae) is important medicinally, distributed in India, Srilanka, Philippines and Indonesia. In India it is distributed in Eastern and Western Ghats and North East India. A deciduous medium sized tree, the pods, seeds, stem and root bark contains many flavones, weak acids and traces of alkaloids (Uddin et al., 2003; Dalal and Rai, 2004). Leaves are emollient and contain anthraquinone and aloe-emodin (Parrotta, 2001; Nakahara et al., 2002). The fruits are used in treating bronchitis, leucoderma, helminthosis etc., (Parrotta, 2001; Dalal and Rai, 2004). The seed extract exhibits antimicrobial, analgesic, antitussive and anti-inflammatory properties (Rasadah et al., 1998). In general, roots are used as astringent and for the treatment of tuberculosis (Bhattacharje, 2000). Decoction of root bark is effective to cure nasopharyngeal cancer (Mao, 2002). In India roots are used in Ayurvedic preparation called "Dasamoola" considered to be an astringent, anti-inflammatory, antihelminthic, antibronchitic, antileucodermatic, antirheumatic, antianorexic and for treatment of leprosy etc., (Manonmani et al., 1995).

According to the report of task force on conservation and sustainable use of medicinal plants, Planning commission, Government of India (2000), the estimated demand of *O. indicum* in Southern India is 500 kg per annum. It is scarce due to escalating demand in pharmaceutical industry, slash and burn cultivation and habitat destruction. The existence of *O. indicum* in natural population is highly threatened and has been categorized as vulnerable by the government of India (Ravikumar and Ved, 2000).

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Abbreviations: CTAB, Hexadecyltrimethylammonium bromide; *Taq, Thermus aquaticus*; PVP, Polyvinylpyrrolidone; TBE, Tris borate-EDTA.

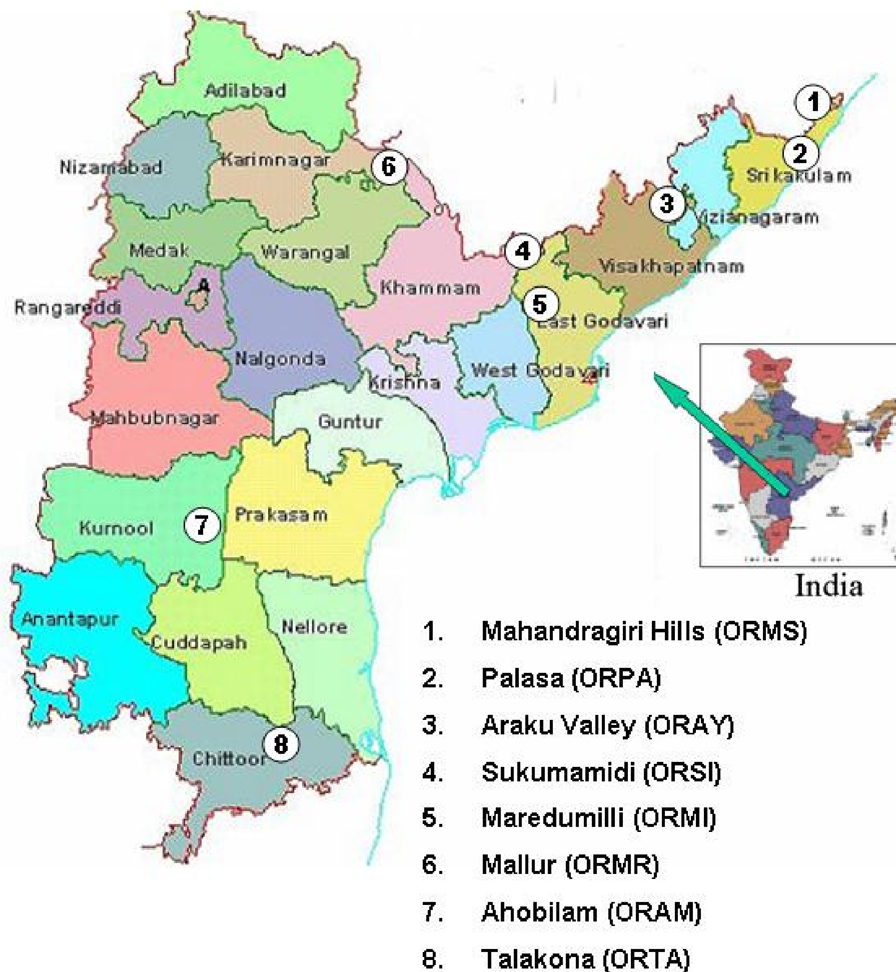

Figure 1. Sampling locations of oroxylum indicum from various districts of Andhra Pradesh,India.

Frankel (1974) opined genetic variation is essential for the long term survival of species, and it is a critical feature in conservation. *Ex-situ* conservation will help to maintain the population of the redlisted species by facilitating release back to nature for native habitat restoration (Li et al., 2002). Therefore, tracing successfully adapted variants at genetic level of *O. indicum* is of immediate necessity for their long-term preservation of these species.

For efficient conservation and management, the genetic composition of the species in different geographic locations needs to be assessed. Due to technical simplicity and speed, RAPD methodology has been used for diversity analysis in many red listed plants (Li et al., 2002; Fu et al., 2003; Padmalatha and Prasad, 2006 a, b; 2007). The objective of the present study is to assess genetic diversity among the accessions using RAPD markers to provide genetic data and a theoretical basis for protection of the species. Hence, an attempt was made to investigate variation among eight accessions of *O. indicum* by using RAPD markers. RAPD markers are based on the amplification of unknown DNA sequences using single, short, random oligonucleotide primers, therefore, RAPD polymorphism is the reflection of variation of the whole genomic DNA, and would be a better parameter to measure the pattern of genetic diversity of the rare and endangered plants.

MATERIAL AND METHODS

Study species and population sampling

An extensive field survey was carried out throughout Andhra Pradesh (A.P), India and a total of eight accessions were collected (Figure 1). The germplasm in the form of plants (273 in number) was maintained in the field experimental site in University of Hyderabad, Andhra Pradesh (A.P) (Table 1) and seeds were stored in seed bank at 4°C. These accessions represent the overall distribution in A.P. The sampling localities are Mahendragiri Hills

Table 1. Population of *Oroxylum indicum* used as sources of DNA.

S/N	Locality	District name	Type of germplasm collected		No. of plants from different accession
1	MahendragiriHills (ORMS)	Srikakulam	Plants	Seeds	25
2	Palasa (ORPA)	Srikakulam	Plants	-	49
3	Araku Valley (ORAY)	Visakhapatnam	Plants	Seeds	21
4	Sukumamidi (ORSI)	Khammam	Plants	Seeds	105
5	Maredumilli (ORMI)	East Godavari	Plants	Seeds	59
6	Mallur (ORMR)	Warangal	Plants	-	3
7	Ahobilam (ORAM)	Kurnool	Plants	-	4
8	Talakona (ORTA)	Chittoor	Plants	-	7

(ORMS) (Srikakulam), Palasa (ORPA) (Srikakulam), Araku valley (ORAY) (Visakhapatnam), Sukumamidi (ORSI) (Khammam), Maredumilli (ORMI) (East Godavari), Mallur (ORMR) (Warangal), Ahobilam (ORAM) (Kurnool) and Talakona (ORTA) (Chittoor). Accessions are separated geographically by a minimum distance of 50 km. The distance between adjacent samples was at least 10 m to increase possibility of detecting the variation potential of each accession. The accession codes and district names are written in parentheses. Since it is a vulnerable plant, distributed sparsely in few locations of A.P as mentioned above, the accession number could not be improved further.

Genomic DNA isolation and RAPD PCR amplification

Young leaf tissue (250 mg) was used for extracting total genomic DNA by following the protocol of Khanuja et al. (1999) with few modifications. The leaf tissue was powdered in liquid nitrogen and immediately transferred to a micro-centrifuge tube containing 1 ml of extraction buffer (100 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 1.5 M NaCl, 2% CTAB, 0.2% β-mercaptoethanol (v/v) and 1% PVP (w/v) (added immediately before use) and mixed well to form a slurry and incubated at 65°C for 60 - 90 min with slow shaking for every 15 min. An equal volume of chloroform: isoamylalcohol (24:1 v/v) was added to the extract prior to centrifugation at 10, 000 rpm for 10 min at room temperature (RT). To the supernatant equal volumes of isopropanol and 2 mM sodium acetate was added and incubated at –20°C for a minimum of 30 min followed by centrifugation at 10, 000 rpm for 10 min at RT. The pellet was washed with 70% ethanol by centrifuging at 10, 000 rpm for 8 min, dried and Tris- EDTA (TE) and 3 µl of RNase A (10 µg/ml) was added to the supernatant and incubated for 1 h at 37°C, followed by extraction twice with chloroform:Iso-amylalcohol (24:1) and centrifuging at 12, 000 rpm for 10 min at RT. To the supernatant cold absolute ethanol was added and DNA was precipitated by centrifuging at 12, 000 rpm for 10 min followed by 80% ethanol washing twice, air dried and the DNA was dissolved in 100 µl of TE buffer. DNA quality and quantity were evaluated spectrophotometrically at OD 260/280 nm and by visual assessment of band intensities on 0.8% agarose gel in comparison to Lambda DNA marker. DNA samples were diluted with sterile Milli Q water to 50 ng/µl for further use in RAPDs.

DNA amplification was performed in a MJ Research Inc. thermalcycler using RAPD markers. The 40 primers were selected from the primer Kits A and C provided by (Operon technologies Inc, Alameda, CA). Genomic DNA (50 ng) was amplified via the PCR reaction using 25 µl reaction volume under the following conditions: 1X PCR buffer (10 mM Tris-HCl and 50 mM KCl (pH 8.3), 3 mM $MgCl_2$ (Invitrogen Life Technologies, India), 0.2 mM dNTP mix (Genetix, New Delhi, India), 0.5 µM of primer and 1U *Taq* polymerase (Invitrogen Life Technologies, India) and programmed for an initial denaturation of 3 min at 94°C followed by 30 cycles of 45 s at 94°C, 1 min at 37°C and 1 min at 72°C and finally a 7 min extension at 72°C and a hold temperature at 4°C. Negative controls were also run in the experiments. All the experiments were repeated thrice to ensure the reproducibility. Amplified DNA fragments were separated by electrophoresis at 60 V in 1X TBE buffer for 3 - 4 h on 2% agarose gels stained with ethidium bromide and photographed by the Gel documentation system (LTF Labortechnik, Germany).

RAPD data analysis and scoring

Since RAPD is a dominant marker, we assumed that each band represented the phenotype at a single biallelic locus. Therefore amplified fragments were scored for the presence (1) and absence (0) of homologues bands. The resulting presence/absence (no. of bands) data matrix of the RAPD phenotypes was analyzed to compute the Dice similarity coefficient (Dice, 1945) using (Numerical Taxonomy and Multivariate Analysis System) NTSYS-pc version 2.02 j software (Rohlf, 1998).

RESULTS

RAPD data analysis

Analysis of eight accessions of *O. indicum* revealed 49.61% of polymorphism. A total of 387 bands were scored for the 40 RAPD primers out of which 188 bands are polymorphic with number of bands ranging from 4 to 20, corresponding to an average of 9.6 bands per primer (Figure 2). Percentage of polymorphic bands ranged from 0 to 100% (Table 2). The levels of genetic similarity within accessions ranged from 0.8077 to 0.9575. The mean value of genetic distance (GD) among the accessions is 0.8693. The minimum genetic similarity of 0.8077 is exhibited between the accessions collected from ORAM and ORPA and maximum genetic similarity of 0.9575 belongs to accessions from ORMI and ORAY. The values of GDs calculated which are grouped together in cluster analysis irrespective of the geographical distances are for

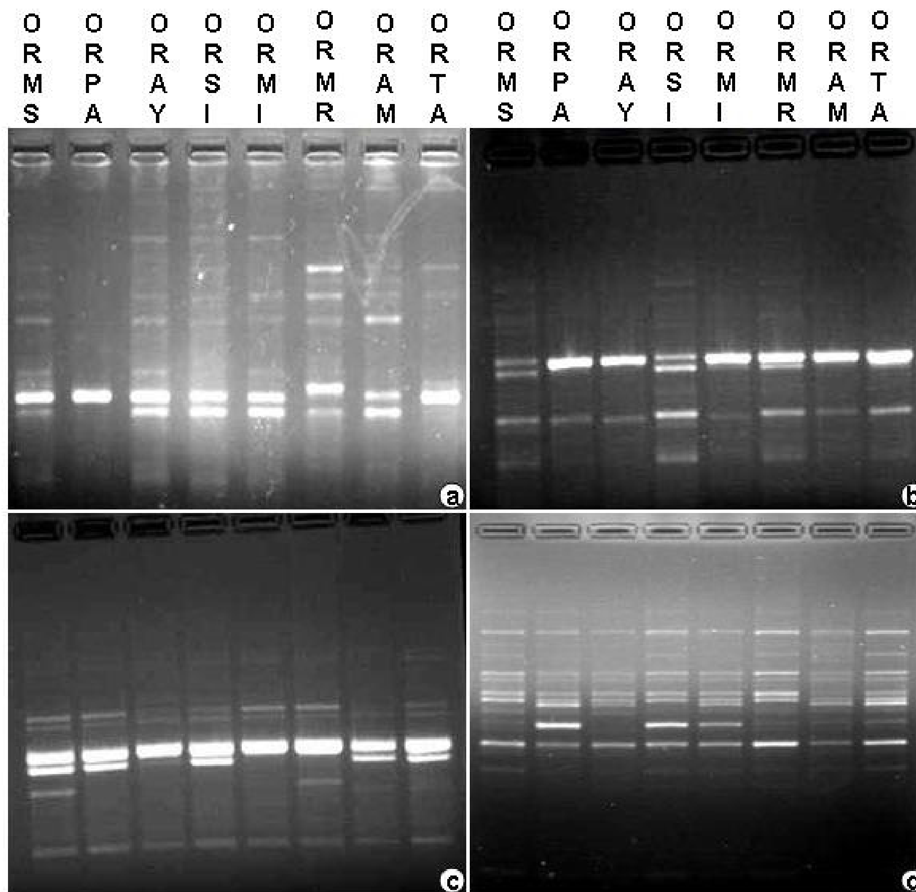

Figure 2. a) RAPD profiles of 8 accessions of *O. indicum* using primer a) OPA-15 (5' TTCCGAACCC 3'), b) OPC-01 (5' TTCGAGCCAG 3'), c) OPC-04 (5' CCGCATCTAC 3') and d) OPC-16 (5' GACGGATCAG 3'). Polymorphic markers were generated with all the accessions.

the accessions collected from ORMS and ORMR which are 0.898; ORAY and ORMI, 0.957; ORPA and ORTA, 0.860. The primer with maximum number of polymorphic bands is OPA-05 and OPA-10 (10 bands) and minimum with OPA-18 and OPC-07 (1 band). The primer OPC -19 exhibits monomorphism. The overall similarity coefficient for all accessions was averaged to 0.8693 (Table 3) and the dendrogram was drawn, based on the GD from RAPD data.

In the dendrogram, the eight collected accessions were divided into two major groups based on the GD. The first group is again classified into 3 sub groups, where the first group includes the accessions collected from ORMS and ORMR. The second subgroup from ORAY and ORMI, which are genetically similar and showed similarity with accessions from ORSI. The third subgroup includes accessions from ORPA and ORTA. The fourth subgroup consists of accession collected from ORAM, which formed a single separate group (Figure 3). The range of the amplified fragments is from 200 –1, 800 bp.

DISCUSSION

The genetic differentiation of accessions of *O. indicum* could broadly be explained as a result of abiotic (geographical, e.g., hydrographic connections, or climatic differentiation. e.g., annual rainfall differences) and biotic (pollination between populations and seed dispersal) factors. The percentage of polymorphism i.e., 49.61 was higher in comparison to other endangered plants, e.g. *Lactoris fernandeziana* (Lactoridaceae) (24.5%) (Brauner et al., 1992), *Paeonia suffruticosa* (22.5%) and *Paeonia rockii* (27.6%) (Pei et al., 1995), *Cathaya argyrophylla* (32%) (Wang et al., 1996), and *Dacydium pierrei* (33.3%) (Su et al., 1999). This shows that the species genetic diversity by itself is low, but relatively higher when compared to other endangered species as stated above and it should be able to adapt to the environmental variation.

High diversity would be expected in populations of *O. indicum* since pollination occurs by bats and black

Table 2. Polymorphism in *O. indicum* using forty RAPD markers.

S/N	Code	5' to 3'	No. of bands	No. of polymorphic bands	Polymorphism (%)
1	OPA-01	CAGGCCCTTC	8	3	37.5
2	OPA-02	TGCCGAGCTG	5	3	60
3	OPA-03	AGTCAGCCAC	9	5	55.5
4	OPA-04	AATCGGGCTG	13	7	53.8
5	OPA-05	AGGGGTCTTG	13	10	76.9
6	OPA-06	GGTCCCTGAC	5	3	60
7	OPA-07	GAAACGGGTG	10	6	60
8	OPA-08	GTGACGTAGG	11	8	72.7
9	OPA-09	GGGTAACGCC	11	9	81.8
10	OPA-10	GTGATCGCAG	11	10	90.9
11	OPA-11	CAATCGCCGT	6	4	66.6
12	OPA-12	TCGGCGATAG	8	2	25
13	OPA-13	CAGCACCCAC	6	2	33.3
14	OPA-14	TCTGTGCTGG	11	8	72.7
15	OPA-15	TTCCGAACCC	7	6	85.7
16	OPA-16	AGCCAGCGAA	7	2	28.5
17	OPA-17	GACCGCTTGT	7	4	57.1
18	OPA-18	AGGTGACCGT	11	1	9.0
19	OPA-19	CAAACGTCGG	5	3	60
20	OPA-20	GTTGCGATCC	11	8	72.7
21	OPC-01	TTCGAGCCAG	4	2	50
22	OPC-02	GTGAGGCGTC	15	9	60
23	OPC-03	GGGGGTCTTT	8	3	37.5
24	OPC-04	CCGCATCTAC	6	3	50
25	OPC-05	GATGACCGCC	20	9	45
26	OPC-06	GAACGGACTC	8	2	25
27	OPC-07	GTCCCGACGA	10	1	10
28	OPC-08	TGGACCGGTG	10	6	60
29	OPC-09	CTCACCGTCC	10	3	30
30	OPC-10	TGTCTGGGTG	9	5	55.5
31	OPC-11	AAAGCTGCGG	9	5	55.5
32	OPC-12	TGTCATCCCC	13	4	30.7
33	OPC-13	AAGCCTCGTC	14	7	50
34	OPC-14	TGCGTGCTTG	9	3	33.3
35	OPC-15	GACGGATCAG	15	4	26.6
36	OPC-16	CACACTCCAG	11	3	27.2
37	OPC-17	TTCCCCCCAG	6	6	100
38	OPC-18	TGAGTGGGTG	10	4	40
39	OPC-19	GTTGCCAGCC	12	0	0
40	OPC-20	ACTTCGCCAC	13	5	38.4

ants, dispersal of winged seeds by wind, habitat changes and larger population size in different locations of A.P. Low genetic variation was observed, which might be due to self pollinated nature of the plant. It may occur due to restricted distribution in a particular area, less possibility of introgressions during evolution, non-effective gene flow, low fecundity, low pollen flow, local selection pressures (environment and struggle for existence) and inbreeding systems (Loveless and Hamrick, 1984; Loveless, 1992). Apart from the above mentioned, biotic factors like human interference, habitat destruction, commercial exploitation, etc., also attribute to high genetic similarity. It is, therefore, a good strategy to protect more of their habitats.

Table 3. Similarity matrix generated from Dice coefficient estimate based on the number of shared fragments.

	ORMS	ORPA	ORAY	ORSI	ORMI	ORMR	ORAM	ORTA
ORMS	1.0000							
ORPA	0.8637	1.0000						
ORAY	0.8928	0.8585	1.0000					
ORSI	0.8971	0.8527	0.9060	1.0000				
ORMI	0.9027	0.8724	0.9575	0.9096	1.0000			
ORMR	0.8987	0.8519	0.8849	0.8957	0.8917	1.0000		
ORAM	0.8227	0.8077	0.8542	0.8448	0.8683	0.8135	1.0000	
ORTA	0.8621	0.8600	0.8376	0.8417	0.8511	0.8828	0.8590	1.0000

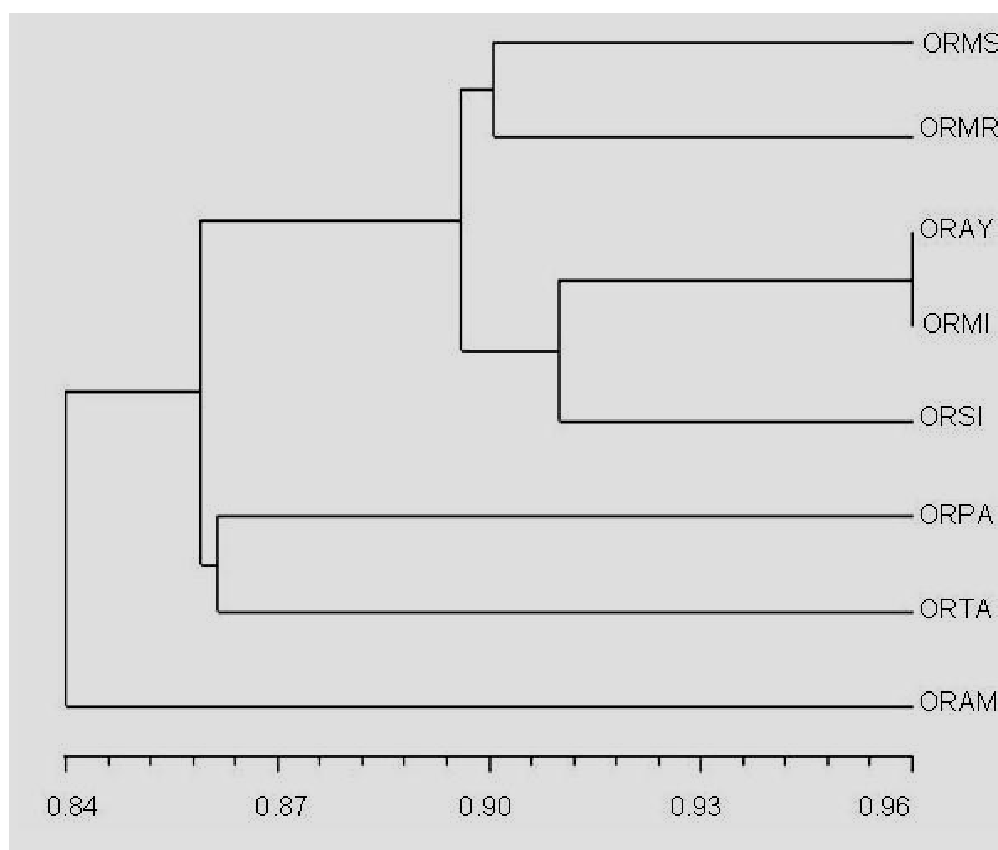

Figure 3. UPGMA cluster analysis of RAPD data for 8 different accessions of *O. indicum*.

The range of GD is 0.8077 to 0.9575 from which it is evident that GD showed no correlation with geographical distances between the accessions, negating a simple isolation by distance mode and it clearly depicts the inbreeding nature of the accessions except in few (Eg. Ahobilam and Maredumilli). Cluster analysis based on Dice coefficient revealed two major groups. The significant degree of variation between the accessions collected from Ahobilam (ORAM) and Palasa (ORPA) according to similarity matrix reveals maximum diversity locations ORAM and ORPA is around 600 Km. There is a close similarity of 95% between the accessions from Araku valley (ORAY) and Maredumilli (ORMI) from the similarity matrix and the dendrogram which clearly depict that genetically they are more or less similar where the distance from Araku valley to Maredumilli is around 200 Km which might have shared few genetic traits with the accession collected from (Sukumamidi) ORSI as all the three formed a single sub group. The geographical distance between Sukumamidi and Maredumilli is around

50 Km which is in accordance with the genetic analysis. The accessions collected from Mahendragiri (ORMS) and Mallur (ORMR) formed a different sub group, which shared a genetic similarity of 89% among themselves. The distance between these two locations is approximately 550 Km, but there is higher genetic similarity within these two accessions as observed in the dendrogram. The accessions collected from Palasa (ORPA) and Talakona (ORTA) exhibited a genetic similarity of 86%, though wide variation is observed between the geographical distances as the distance between Palasa and Talakona is around 1000 Km.

The accession collected from Ahobilam (ORAM) varies significantly when compared to other accessions collected from various locations which have to be investigated further as it is falling in an entirely different group. Such observations have been reported previously in *Hordeum spontaneum* populations by Dawson et al. (1993). It can be inferred that from the accessions grouped into similar groups there is an effective gene flow in those locations. Whereas between the accessions collected from Ahobilam (ORAM) when compared with other accessions the gene flow is less. So also for the accessions from Ahobilam (ORAM) and Maredumilli (ORMI) gene flow may be less and hence are highly divergent when compared to other accessions.

It can be predicted that every finite population may not experience genetic drift, but the effect will become more pronounced as population size increases and in due course there might be low genetic variation. The other cause of low genetic variation might be due to the vulnerable nature of the plant as during evolution inbreeding may occur which also is in accordance with the field observation as the population lies very closer. The mode of natural propagation is also by suckers apart from seeds which may also aid in self pollination to some extent. As mentioned above selfing predominates in nature as in *O. indicum*, but cross pollination also might occur in a negligible rate.

Only with a firm grasp of the genetic structure and the diversity grade of populations in rare and endangered species can we make an efficacious measurement and strategy of protecting them. For a species with limited gene flow and over 50% variation among accessions, it is necessary to collect samples from at least six accessions in order to conserve 95% of the genetic diversity of the species. For a species with only 20% variation among accessions, the samples taken from two accessions are enough to get the same results above (Pei et al., 1995; Yun et al., 1998). The results of RAPD show that we need to take individuals from more different populations if we are to construct an artificial conservation area so as to preserve their diversity for the future.

Conservation implication

The overall genetic diversity of a taxon has great implications for its long-term survival and continued evolution (Avise and Hamrick, 1996). Therefore, knowledge of the levels and distribution of genetic diversity is important for designing conservation strategies for threatened and endangered species (Hamrick, 1983; Hamrick and Godt, 1989; Francisco-Ortega et al., 2000). Based on the information available for *O. indicum*, two alternative conservation strategies can be proposed.

1. *In situ* conservation plan that defines areas free from significant disruption for at least the genetically most diverse accessions (ORAM). This would guarantee the maintenance of most of the species' genetic variation. However, because the observed genetic differentiation among accessions of *O. indicum* is high and little gene flow appears to exist among the accessions, management for the conservation of genetic variability in this species not only aim to preserve large accessions collected from ORAM but also many of the small accessions collected from ORAY and ORMI.

2. *Ex situ* conservation of the accessions accordingly based on the data on RAPD markers would fulfill the objective of capturing most of the detected genetic variability. Although seed set is high, we have observed low recruitment of seedlings in natural conditions in *O. indicum*. Therefore, any method of *ex situ* conservation that anticipates reintroduction must optimize the survival of seedlings to assure the viability of these accessions. Specifically we propose an *ex situ* conservation strategy based on collection of seeds and plants, propagation and maintenance in Botanical garden of University of Hyderabad. *In vivo* experiments have proved higher percentage of germination (80%). Thus a strategy involving extensive seed and plant collection to ensure full sampling of genetic diversity, followed by cultivation in field plots and subsequent reintroduction into the wild seems feasible. Consequently we suggest that care be taken to separate seedlings from different populations and reintroduce them into their original parental localities. In these ways we hope that the future of this medicinally important, severely threatened species will be guaranteed.

There are some indications that regional, habitat, photoperiod and temperature can affect secondary metabolite production (Kämäräinen et al., 2003). Recently studies on differences in oil composition in *Cunila galioides* were done which allowed them to identify different chemotypes (Echeverrigaray et al., 2003). Hence germplasm of *O. indicum,* collected from various parts of A.P may show some variation in terms of secondary metabolites content i.e., different chemotypes.

The genetic similarity among the accessions of *O. indicum* reflected a low level of DNA polymorphism. Based on the field observations of this study and cluster analysis, the accessions belonging to ORMR and ORMS, ORPA and ORTK and ORAY and ORMI share maximum

genetic similarity. Thus any one of those can be conserved which might prove as an effective conservation management practice. For *ex situ* conservation, the germplasm from ORAM can be given the primary importance. Appropriate conservation measures have been initiated in field gene bank in University of Hyderabad campus along with other redlisted medicinal plants collected from Deccan ecoregion (Prasad et al., 2006, 2007). It can also be assumed that the genetic similarity observed may also contribute to the similarity in the composition of secondary metabolites among the collected accessions which can establish a baseline for further studies.

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