BIOLOGIA PLANTARUM 56 (2): 292-300, 2012

Low genetic diversity as revealed by SPAR methods possibly leads to extinction of two critically-endangered and endemic species of *Mantisia*

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Abstract

Mantisia spathulata Schult. and *M. wengeri* Fischer, two critically-endangered, endemic and rare species of the genus *Mantisia* (*Zingiberaceae*), have been rediscovered from Lunglei province of Mizoram, India, after two decades. For sustainable conservation and utilization of the *Mantisia* species, *in vitro* seed and clonal propagation methods have been developed earlier by our research group and plantlets have been reintroduced to their natural habitat for species recovery. To comprehend the plausible reasons for endemism and endangeredness of both the species at DNA level, they were analyzed to assess natural genetic variation using three different polymerase chain reaction (PCR) based DNA markers *viz.* random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR) and directed amplification of minisatellite DNA regions (DAMD), both individually and cumulatively, which are popularly regarded as single primer amplification reaction (SPAR) methods. A total of 107 primers belonging to three SPARs are used which collectively endow low genetic variation (15 and 20 %, respectively) in both *M. spathulata* and *M. wengeri.* The use and efficacy of SPAR methods to reveal the natural genetic variation in *Mantisia* species at intra-specific level has been recorded for the first time. To impede the extinction risk of these two species of genus *Mantisia,* large scale conservation strategies including *in situ* and *ex situ* conservation are recommended.

Additional key words: conservation, DAMD, ISSR, RAPD, species reintroduction.

Introduction

The genus *Mantisia* is endemic to hilly areas of the Northeastern India, Myanmar and Bangladesh (Dam *et al.* 1997, Rahman and Yusuf 2002, Tandon *et al.* 2007, Bhowmik *et al.* 2009) and comprises only five species, *i.e. Mantisia wengeri*, *M. radicalis*, *M. spathulata, M. wardii* and *M. salarkhanii*. It represents the smallest genus of family *Zingiberaceae*. *M. spathulata* and *M. wengeri* are two critically-endangered species and endemic to Mizoram, a Northeastern state of India (Bhowmik *et al.* 2009). *Mantisia* species are annual or perennial herbs (Bhowmik *et al.* 2009) and aerial shoots of these species used to appear after the development of inflorescence with numerous splendid flowers (Rahman and Yusuf 2002). Due to natural calamities and anthropogenic activities, *M. spathulata* and *M. wengeri* have become critically-endangered in the natural habitat (Ganeshaiah 2005, Tandon *et al.* 2007, Bhowmik *et al.* 2009, 2010a, Sharma *et al.* 2011b) and are listed in the Red Book of rare and endangered Indian plants (www.envfor.nic.in/bsi/research.html). Few reports are available for the phylogenetic analysis related to *Mantisia* and allied genus *Globba* (Takano and Okada 2003, Kress *et al.* 2001). Attempts for conservation through rapid *in vitro* clonal propagation as well as seed germination have been made by our research group earlier (Tandon *et al.* 2007, Bhowmik *et al.* 2009*,* 2010a,b). *In vitro* raised plantlets of both the species after ensuring genetic fidelity have been reintroduced in their natural habitat for

Received 1 August 2010, *accepted* 7 March 2011.

Abbreviations: AFLP - amplified fragment length polymorphism; DAMD - directed amplification of minisatellite DNA regions; ISSR - inter simple sequence repeat; NJ - neighbour-joining; PCR - polymerase chain reaction; RAPD - random amplified polymorphic DNA; RFLP - restriction fragment length polymorphism; SPAR - single primer amplification reaction; UPGMA - unweighted pair-group method with arithmetic averages

Acknowledgement: The present work is supported by a grant from University Grants Commission, Government of India, New Delhi, through University with Potential for Excellence (UPE) - Bioscience program. Sincere thanks are due to Head and all members of Plant Biotechnology Laboratories of Department of Biotechnology and Bioinformatics and Department of Botany, NEHU, Shillong for their constant encouragement and help.

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conservation under the species recovery program (Bhowmik *et al.* 2009). This rarity of the genus *Mantisia* has reached such a level that only few representatives are available. Hence, an effective strategy was a prerequisite to analyze the genetic variation among these plants in natural habitat and to evaluate the reason for the rarity/endemism using molecular marker(s) based approaches.

Rare and endangered species are susceptible to loss of genetic variation through genetic drift in small populations (Xue *et al.* 2004). Successful management of many rare plant populations has been greatly improved by genetic data (Ellstrand and Elam 1993). The gathering of data on genetic structure/variation of rare species has become a common prelude to conservation planning (Archibald *et al.* 2001). Several DNA-based marker systems such as restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites are available now, which can precisely assess the degree of genetic diversity in plant species (Beharav *et al.* 2010, Kang *et al.* 2010, Ma *et al.* 2010, Pei *et al.* 2010, Sikdar *et al.* 2010, Viana *et al.* 2010). In the recent years, the PCR based single primer amplification reaction (SPAR) methods are gaining prominence as effective tools for genetic diversity studies in plants and they collectively provide a comprehensive description of the nature and extent of the diversity (Bhattacharya *et al.* 2005, 2010, Ranade *et al.* 2009, Sharma *et al.* 2010, 2011a). PCR based marker approaches involving single primer for amplification reactions are commonly used in diversity analysis of higher plants which mainly include directed amplification of minisatellite DNA regions (DAMD; Heath *et al.* 1993), inter simple sequence repeat (ISSR; Gupta *et al.* 1994) and RAPD (Welsh and McClelland 1990, Williams *et al.* 1990).

There is an urgent need for thorough exploration and exploitation of the available genetic resources in both *M. spathulata* and *M. wengeri,* so as to accomplish sustainable conservation and utilization. Hence, the present investigation has been undertaken with the main objective of understanding the existing genetic diversity to define and characterize the variation at inter- and intra-specific levels.

Materials and methods

Ten groups of *Mantisia spathulata* Schult. and *Maninsia wengeri* Fischer plants were collected from their natural habitat located at Lunglei, Mizoram, India, in May - June 2007 and grown in pots in a glasshouse of North-Eastern Hill University, Shillong, India for 20 - 24 weeks. Frozen leaves were ground and powdered in a pre-chilled mortar using liquid nitrogen, and the DNA was then extracted following the modified cetyltrimethyl-ammonium bromide (CTAB) method. The DNA extracted from the plant material, purified for protein fraction, treated with RNase A, was re-precipitated with pre-chilled absolute ethanol and subsequently dissolved in Tris-EDTA (TE) buffer. The quality of DNA was checked by gel electrophoresis with 0.85 % (m/v) agarose in 1× Tris-acetate-EDTA (TAE).

Six kits (OPA, OPB, OPC, OPD, OPAA and OPBA) each comprising 20 random decamer primers were procured form *Operon Technologies*, Alameda, CA, USA. Thirty ISSR primers and eight DAMD primers were custom synthesized from *Metabion*, Martinsried, Germany (Table 1).

Varying concentrations of template DNA (20, 30, 40, 50 and 60 ng), Taq DNA polymerase (0.5 - 2 U; *Bangalore Genei*, Bengaluru, India)) and $MgCl_2$ (1 - 5 mM) were used to optimize the PCR reaction. The best results were obtained with 50 ng DNA, 1 U Taq DNA polymerase and 1.5 mM of $MgCl_2$.

Three randomly selected collections were chosen for primer survey and screening from both the *Mantisia* species. From the above mentioned kits 120 RAPD, 30 ISSR and 8 DAMD primers were assayed with these three accessions to identify primers that were reproducible and generate the most polymorphic patterns. Following the results of initial primer screening experiments, 30 RAPD decamer primers, 20 ISSR and 6 DAMD primers for *M. spathulata* and 28 RAPD decamer primers, 17 ISSR and six DAMD primers for *M. wengeri* (Table 1) were selected for final amplification programme under optimized conditions mentioned above. All further reactions were performed in 0.025 cm^3 final volume and contained 50 ng template DNA, 20 pmol (RAPD) or 40 pmol (ISSR and DAMD), 200 µM each dNTP, 1.5 mM $MgCl_2$ and 1 U Taq DNA polymerase. Reaction conditions in a thermal cycler *2720* (*Applied Biosystems*, Foster City, CA, USA) were: 94 °C for 3 min followed by 44 cycles at 94 °C for 1 min, 35 °C (in case of ISSR and DAMD amplification, annealing temperature varied according to primer's Tm ranging from 45 to 55 °C) for 1 min 30 s, and at 72 °C for 2 min, with a final extension at 72 °C for 7 min. After completion of the amplification, 0.0025 cm^3 10× blue dye was added to the samples, and the amplified DNA was analyzed on 1.5 % (m/v) agarose gel in 1× TAE buffer at 65 - 70 V for 3 - 4 h.

The amplification products were scored across all the samples and recorded as present (1) or absent (0). Faint bands were not considered for final scoring. Molecular mass of the bands were estimated by using *Gene Rular* 500 bp DNA ladder (*MBI Fermantas*, Cambridge, UK) as standards. All amplifications were repeated at least twice and only reproducible bands were considered for analysis. The data were scored individually, first for all the primers in a SPAR method and subsequently the data sets for all the three methods used. A pair-wise matrix of

similarity between genotypes was determined cumulatively for all three methods using Dice coefficient (Dice 1945) by the *NTSYS-pc*, version *2.02k* (*Applied Biosystems*). Consensus values were recorded discretely for each SPAR which produces a consensus tree and computes consensus indices from two or more rooted labeled trees. Using the *NTSYS-pc* software, co-phenetic analysis for matrix comparison, and *MxComp* analyses to calculate *r*-value were performed, which can be used as a measure of goodness of fit and correlated with reliability of the SPAR methods, either collectively or independently.

Results

***Mantisia spathulata*:** A total of 272 amplification products using 56 single primers were scored, which exhibited an overall 46.69 % polymorphism and 4.77 average numbers of amplicons collectively (Table 1). RAPD method revealed the average number of amplification products formed was 4.8 with a maximum of 11, rendered an overall 36.80 % polymorphism individually (Table 1). ISSR and DAMD methods showed in average 4.6 and 6.0 amplicons formed and 54.34 and 66.66 % polymorphism, respectively, when calculated individually. The size of the amplification products varied in case of each SPAR method and the range was 0.3 to 2.5 kb. Fig. 1 and Table 1 are illustrative of the extent of polymorphism observed among the 10 collections of *M. spathulata.*

The NJ tree (Fig. 2*A*) was constructed by using the

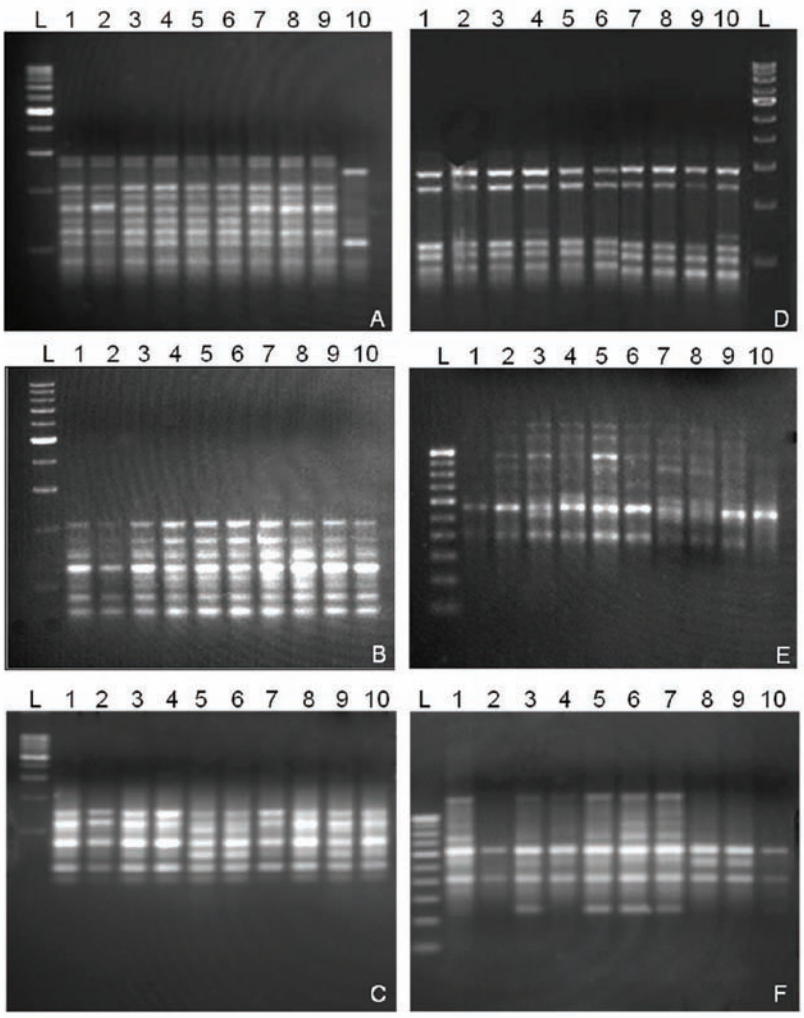

Fig. 1. Typical SPAR profiles obtained with one RAPD primer (OPBA-7, *A*), one ISSR primer (I-811, *B*) and one DAMD primer (HBV, *C*) for all 10 collections of *M. spathulata* and profiles of one RAPD primer (OPA-4, *D*), one ISSR primer (I-835, *E*) and one DAMD primer (INS, *F*) for all 10 collections of *M. wengeri*. All profiles were resolved in 1.5 % agarose gels in TAE buffer. The *lanes* L represent known DNA fragment size markers. The other *lanes* are marked with collection numbers for both *M. spathulata* and *M. wengeri*.

Table 1. RAPD, ISSR and DAMD primers used in the present study and the extent of polymorphism in both *M. spathulata* and *M. wengeri*.

Species	Method	Sl No.	Primer name	Primer sequence (5'-3')	Total number of bands	Number of polymorphic bands	Polymorphism [%]
Mantisia spathulata	RAPD	1.	OPA-4	AATCGGGCTG	5	2	40.00
		2.	OPA-5	AGGGGTCTTG	4	1	25.00
		3.	OPA-8	GTGACGTAGG	4	2	50.00
		4.	OPA-9	GGGTAACGCC	4	0	0.00
		5.	OPA-10	GTGATCGCAG	3	1	33.33
		6.	OPB-7	GGTGACGCAG	3	1	33.33
		7.	OPB-9	TGGGGGACTC	6	2	33.33
		8.	OPB-10	CTGCTGGGAC	3	1	33.33
		9.	OPC-2	GTGAGGCGTC	4	4	100.00
		10.	OPC-4	CCGCATCTAC	6	3	50.00
		11.	OPC-6	GAACGGACTC	5	3	60.00
		12.	OPC-15	GACGGATCAG	4	4	100.00
		13.	OPD-3	GTCGCCGTCA	4	0	0.00
		14.	OPD-5	TGAGCGGACA	4	0	0.00
		15.	OPAA-7	CTACGCTCAC	6	0	0.00
		16.	OPAA-9	AGATGGGCAG	4	1	25.00
		17.	OPAA-14	AACGGGCCAA	6	0	50.00
		18.	OPAA-15	ACGGAAGCCC	3	3	100.00
		19.	OPAA-17	GAGCCCGACT	6	0	0.00
		20.	OPAA-18	TGGTCCAGCC	5	2	40.00
		21.	OPBA-3	GTGCGAGAAC	7	0	0.00
		22.	OPBA-4	TCCTAGGCTC	7	6	85.72
		23.	OPBA-5	TGCGTTCCAC	5	4	80.00
		24.	OPBA-6	GGACGACCGT	5	3	60.00
		25.	OPBA-7	GGGTCGCATC	11	5	45.45
		26.	OPBA-8	CCACAGCCGA	5	3	60.00
		27.	OPBA-12	TGTTGGGCAC	4	0	0.00
		28.	OPBA-13	AGGGCGAATG	3	0	0.00
		29.	OPBA-15	GAAGACCTGG	5	2	40.00
		30.	OPBA-16	CCACGCATCA	3	0	0.00
	ISSR	31.	I-807	AGAGAGAGAGAGAGAGT	4	0	0.00
		32.	I-808	AGAGAGAGAGAGAGAGC	4	1	25.00
		33.	I-811	GAGAGAGAGAGAGAGAC	8	5	62.50
		34.	I-814B	CTCTCTCTCTCTCTCTA	4	3	75.00
		35.	I-815	CTCTCTCTCTCTCTCTG	4	2	50.00
		36.	I-821	GTGTGTGTGTGTGTGTT	3	2	66.66
		37.	I-825	ACACACACACACACACT	4	0	0.00
		38.	I-827	ACACACACACACACACG	4	2	50.00
		39.	I-828	TGTGTGTGTGTGTGTGA	4	0	0.00
		40.	I-835	AGAGAGAGAGAGAGAGYC	4	2	50.00
		41.	I-840	GAGAGAGAGAGAGAGAYT	5	5	100.00
		42.	I-841	GAGAGAGAGAGAGAGAYC	3	0	0.00
		43.	I-845	CTCTCTCTCTCTCTCTRG	7	7	100.00
		44.	I-853	TCTCTCTCTCTCTCTCAT	3	0	0.00
		45.	I-857	ACACACACACACACACYG	6	6	100.00
		46.	I-864	ATGATGATGATGATGATG	4	0	0.00
		47.	I-79898A	CACACACACACAAC	4	0	0.00
		48.	I-79899A	CACACACACACAAG	3	1	33.33
		49.	I-79899B	CACACACACACAGG	6	6	100.00
		50.	I-HB15	GTGGTGGTGGC	8	8	100.00
	DAMD	51.	HBV	GGTGTAGAGAAGGGGT	7	4	57.14
		52.	HVR	CCTCCTCCCTCCT	8	8	100.00
		53.	M13	GAGGGTGGNGGNTCT	6	2	33.33
		54.	YNZ22	CTCTGGGTGTCGTGC	3	1	33.33
		55.	INS	ACAGGGGTGGGG	6	3	50.00
		56.	33.6	GGAGGTTTTCA	6	6	100.00

Mantisia wengeri	RAPD	57.	OPA-4	AATCGGGCTG	6	1	16.66
		58.	OPA-5	AGGGGTCTTG	7	2	28.57
		59.	OPA-9	GGGTAACGCC	3	3	100.00
		60.	OPA-14	TCTGTGCTGG	3	3	100.00
		61.	OPA-20	GTTGCGATCC	6	6	100.00
		62.	OPC-1	TTCGAGCCAG	2	2	100.00
		63.	OPC-2	GTGAGGCGTC	6	6	100.00
		64.	OPC-6	GAACGGACTC	5	5	100.00
		65.	OPC-15	GACGGATCAG	2	2	100.00
		66.	OPD-2	GGACCCAACC	5	5	100.00
		67.	OPAA-6	GTGGGTGCCA	5	0	0.00
		68.	OPAA-9	AGATGGGCAG	6	4	66.66
		69.	OPAA-10	TGGTCGGGTG	2	0	0.00
		70.	OPAA-11	ACCCGACCTG	3	1	33.33
		71.	OPAA-12	GGACCTCTTG	3	0	0.00
		72.	OPAA-14	AACGGGCCAA	4	1	25.00
		73.	OPAA-15	ACGGAAGCCC	5	1	20.00
		74.	OPAA-16	GGAACCCACA	3	2	66.66
		75.	OPAA-17	GAGCCCGACT	5	2	40.00
		76.	OPAA-18	TGGTCCAGCC	3	1	33.33
		77.	OPBA-3	GTGCGAGAAC	5	4	80.00
		78.	OPBA-5	TGCGTTCCAC	5	0	0.00
		79.	OPBA-8	CCACAGCCGA	5	0	0.00
		80.	OPBA-10	GGACGTTGAG	4	4	100.00
		81.	OPBA-14	TCGGGAGTGG	7	7	100.00
		82.	OPBA-18	CTCGGATGTC	4	2	50.00
		83.	OPBA-19	CCATCCGTTG	2	2	100.00
		84.	OPBA-20	GAGCGCTACC	3	3	100.00
	ISSR	85.	I-807	GAGAGAGAGAGAGAGT	2	2	100.00
		86.	I-808	AGAGAGAGAGAGAGAGC	3	1	33.33
		87.	I-811	GAGAGAGAGAGAGAGAC	3	1	33.33
		88.	I-814A	CTCTCTCTCTCTCTCTTG	5	4	80.00
		89.	I-814B	CTCTCTCTCTCTCTCTA	5	3	60.00
		90.	I-815	CTCTCTCTCTCTCTCTG	3	1	33.33
		91.	I-817	CACACACACACACACAA	3	0	0.00
		92.	I-825	ACACACACACACACACT	3	0	0.00
		93.	I-827	ACACACACACACACACG	4	4	100.00
		94.	I-828	TGTGTGTGTGTGTGTGA	7	6	85.71
		95.	I-835	AGAGAGAGAGAGAGAGYC	9	5	55.55
		96.	I-836	AGAGAGAGAGAGAGAGCA	3	1	33.33
		97.	I-840	GAGAGAGAGAGAGAGAYT	4	2	50.00
		98.	I-841	GAGAGAGAGAGAGAGACC	2	2	100.00
		99.	I-850	GTGTGTGTGTGTGTGTTC	2	0	0.00
		100.	I-857	ACACACACACACACACCG	5	4	80.00
		101.	I-864	ATGATGATGATGATGATG	4	2	50.00
	DAMD	102.	HBV	GGTGTAGAGAAGGGGT	5	5	100.00
		103.	HVR	CCTCCTCCCTCCT	6	6	100.00
		104.	M13	GAGGGTGGNGGNTCT	3	3	100.00
		105.	YNZ22	CTCTGGGTGTCGTGC	7	5	71.42
		106.	INS	ACAGGGGTGGGG	8	7	87.50
		107.	33.6	GGAGGTTTTCA	5	4	80.00

cumulative data of three SPAR methods to analyze the genetic distance in all the 10 collections of *M. spathulata* using Dice coefficients of similarity. Remarkably, the trees generated through both the methods had at least three major clusters marked A, B, and C with large parenthesis in Fig. 2*A*. Cluster A may be further resolved into five sub-clusters *i.e*. A1, which includes only accession MS-1, while sub-cluster A2 comprises three accessions (MS-3, 6 and 7). Interestingly, MS-3 and MS-7 showed identical value of genetic distance with highest genetic similarity. The third sub-cluster A3 consists of MS-4 and MS-8, whereas sub-clusters A4 and A5 consist of MS-5 and MS-9, respectively. The interesting fact which lies with both cluster B and C is that each comprise single accession, *i.e*, MS-2 and MS-10, respectively. The fact also draws support from individual analysis of each SPAR method, which showed similar tendency with MS-2 and MS-10 depicting less similarity among all the collections. The dataset of all the three SPAR methods revealed identical rooted trees,

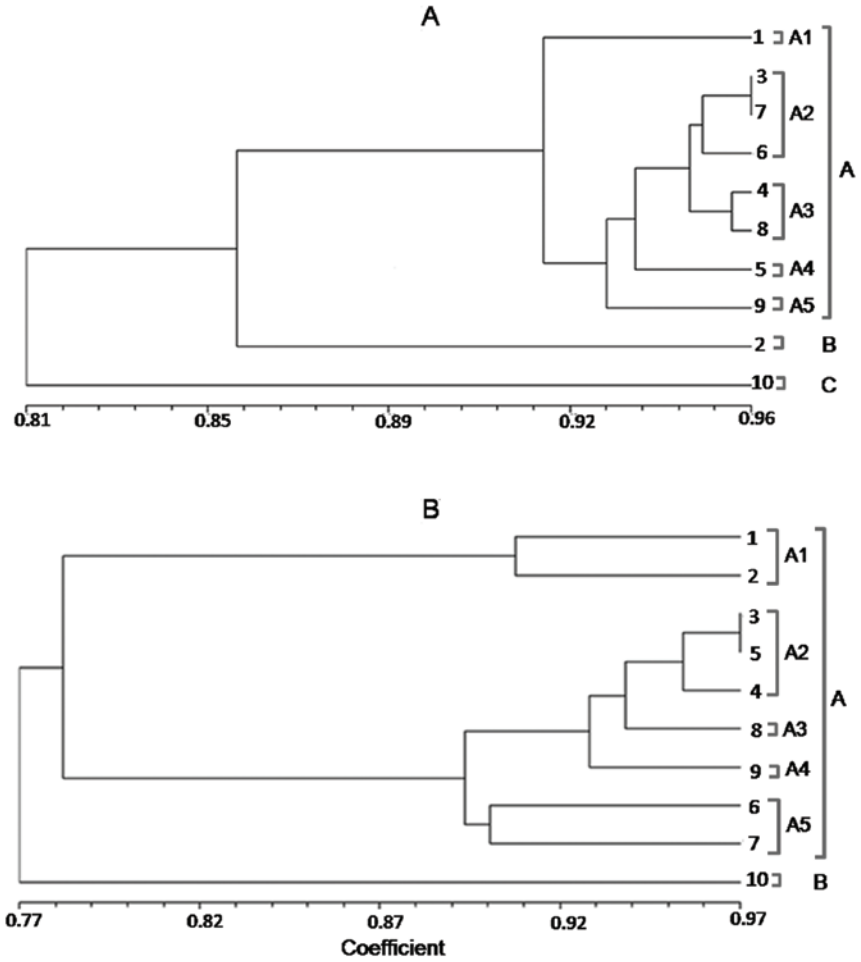

Fig. 2. Cluster analysis of SPAR data in case of the 10 accessions of *M. spathulata* (*A*) and *M. wengeri* (*B*). The NJ tree was generated for cumulative band data by all three SPAR methods, RAPD, ISSR and DAMD. The numbers indicated to the right of the tree are accession numbers for *M. spathulata* and *M. wengeri*. The branch lengths are based on the distance values computed using Dice coefficient of *NTSYS-pc 2.02k* software. The large parenthesis to the right labeled with A-C (in case of *M. spathulata*) and A-B (in case of *M. wengeri*.) are the major clusters while the all inner parenthesis showing sub-clusters within the respective cluster.

when analyzed either individually or collectively, calculated by Dice similarity coefficient that revealed low value ranged between 0.81 - 0.96 (Table 2).

***M. wengeri*:** A total of 220 amplification products using 51 single primers were scored, which exhibited an overall 62.27 % polymorphism and 4.31 average numbers of amplicons collectively (Table 1). RAPD method revealed the average number of amplification products formed was 4.25 with a maximum of 7 with OPA-5 and OPBA-14 with overall 57.98 % polymorphism individually. ISSR and DAMD methods showed the average number of amplicons 3.94 and 5.66 and 56.71 and 88.23 % polymorphism, respectively. The size of the amplification products varied in case of each SPAR method and the range was 0.3 to 2.5 kb. Fig. 1 and Table 1 are illustrative of the extent of polymorphism observed among the 10 collections of *M. wengeri.*

The NJ tree showed two major clusters revealed through Dice similarity coefficients which are marked A and B (Fig. 2*B*). Cluster A resolved five sub-clusters, of which, sub-cluster A1 comprises two collections (MW-1 and 2), sub-cluster A2 includes three collection (MW-3, 4 and 5). In this sub-cluster, MW-3 and MW-5 showed identical value of genetic distance with highest genetic similarity. Notably, sub-cluster A3 and A4 comprises single collections, *i.e*. MW-8 and MW-9, respectively, which can be predicted as totally diverse genotypes. Sub-cluster A5 made up of MW-6 and MW-7 with same genetic distance in the tree. Cluster B had only MW-10. All the SPAR methods, both individually and collectively, resulted in identically rooted trees similarly as observed in *M. spathulata.* Noticeably, low genetic distance values ranging between 0.77 - 0.97 were estimated by Dice coefficient (Table 2).

Comparison of different SPAR methods: To determine the efficiency of the three methods, distance range of each SPAR method was calculated by Dice coefficients, both individually and collectively (Table 2). The genetic

distance among various collections of *M. spathulata* ranged between 0.82 and 0.99 (RAPD), 0.77 and 0.96 (ISSR) and 0.81 and 0.96 (DAMD). The corresponding values were 0.73 - 0.99, 0.82 - 0.97 and 0.35 - 0.98 in *M. wengeri*. Consensus values of SPAR pairs, *i.e.* RAPD + ISSR, RAPD + DAMD and ISSR + DAMD showed moderate results for both the species. Likewise, the co-phenetic value matrix was used to test the goodness of fit of a cluster analysis to the data by using the *MxComp* module to compare the original similarity or dissimilarity matrix that was clustered with the co-phenetic value matrix for both *Mantisia* species. The degree of fit can be interpreted as *r*-value for each pair and it showed the good fit values $0.8 \leq r < 0.9$ for SPAR methods collectively.

Table 2. The various SPAR (RAPD+ISSR+DAMD) methods and details and comparison of their results and analysis computed for all the collections of both *M. spathulata* and *M. wengeri*.

		Number of primers used	Number of bands	Bands/ primer	Number of polymorphic bands	Polymorphic bands/primer	Polymorphism [%]	Distance range
Mantisia spathulata	RAPD	30	144	4.80	53	1.76	36.80	0.82-0.99
	ISSR	20	92	4.60	50	2.50	54.34	0.77-0.96
	DAMD	6	36	6.00	24	4.00	66.66	0.81-0.96
	SPAR	56	272	4.85	127	2.26	46.69	0.81-0.96
Mantisia wengeri	RAPD	28	119	4.25	69	2.46	57.98	0.73-0.99
	ISSR	17	67	3.94	38	2.23	56.71	0.82-0.97
	DAMD	6	34	5.66	30	5.00	88.23	0.35-0.98
	SPAR	51	220	4.31	137	2.68	62.27	0.77-0.97

Discussion

The estimation of genetic diversity at inter- and intra-specific levels based on molecular markers especially SPARs (Bhattacharya *et al.* 2005, Ranade *et al.* 2009, Sharma *et al.* 2010, 2011a) are generally more reliable than estimation based on allozymes and other techniques (Ayres and Ryan 1999). Present investigation deals with three different single primer based PCR amplification methods to analyze natural genetic variation in two rare, endemic and critically-endangered species of genus *Mantisia.* The present investigation revealed the fact that RAPD and DAMD are more effective in detecting polymorphism in case of *M. wengeri* while ISSR was more effective in *M. spathulata*. The three SPAR methods collectively revealed genetic distances ranging between 0.81 - 0.96 in *M. spathulata* and 0.77 - 0.97 in *M. wengeri*. However, the three SPARs collectively revealed higher polymorphism (62.27 %) in *M. wengeri* as compared to *M. spathulata* (46.69 %).

All the results showed low genetic diversity in both *M. spathulata* and *M. wengeri*. Similarly, consensus, co-phenetic and matrix comparison for analysis of degree of fit (*r*-value) for three SPAR methods also generated moderate values which is basically linked with efficacy of the marker system either individually or cumulatively. An interesting and significant aspect of the present investigation has been that RAPDs, ISSR and DAMD, which are all single primer-based amplification strategies, were compared not only individually but also pair-wise with each other. It has been clearly observed that the *r*-value has been highest for ISSR + DAMD pair. Such observations are also depicted in genetic distance values which resemble more to either ISSR and/or DAMD in the present investigation, thereby proving the non-reliability of RAPD marker system to analyze genetic variation when used individually. In the present investigation, DAMD produced highest number of amplicons either in total or polymorphic banding pattern, when compared to RAPD and ISSR (Table 2). The percentage of polymorphism calculated through DAMD markers was higher in comparison to RAPD and ISSR (Table 2), also prove the DAMD to be best suited marker system for determining the genetic variation of these species. Molecular data obtained through PCR based molecular markers can directly be linked with conservation genetics of small, rare and endangered populations (Lande 1988, Frankham 1995, Xue *et al.* 2004) including inbreeding depression as well as out-breeding complications, accumulation of deleterious mutations, loss of genetic variation, genetic adaptation to domestication and its effects on reintroduction success, insect pollination, loss of self-compatibility and taxonomic uncertainties as well as interrogation are some of the factors for rarity, endangerment and endemism, ultimately leading to extinction of the plant species (Frankham 1995). Species with low genetic variation would be expected to have reduced ability to cope up with environmental alterations during evolution (Frankham 1995).

Various reports suggests that natural propagation through seeds in many rare and endangered plants is limited due to many factors such as seed dormancy (Rathore *et al.* 1991), poor seed viability (Sudha and Seeni 1996) and little or no seed production (Agrawal

et al. 1991, McKently and Adams 1994). Recently, Bhowmik *et al.* (2010a) reported comparatively low *in vivo* germination of seeds for *M. spathulata* and *M. wengeri* (20 and 24 %, respectively). Other factors like, squat cross-pollination, entomophily (Bhowmik 2009), imbalanced meiotic events (Sharma *et al.* 2011b), low karyo-morphological variation also depicts the confirma-tion of rarity and endangered status of both the species of genus *Mantisia*.

All measures in the present study aimed at the reintroduction and recovery of the species for sustainable restoration of the allelic composition/heterozygosity in genetic constitution of each species /populations of genus *Mantisia.* Earlier, various attempts have been made by our research group for sustainable conservation and recovery of both the species of genus *Mantisia* through mass propagation and *in vitro* clonal propagation techniques (Tandon *et al.* 2007, Bhowmik *et al.* 2009, 2010b). Recently, our research group reported significant enhancement of seed germination through *in vitro* techniques (Bhowmik *et al.* 2010a). A maximum of 91 and 97 % germination was recorded for *M. spathulata* and *M. wengeri* within two weeks in MS medium supplemented with 4.3 and 7.2 μM GA_3, respectively (Bhowmik *et al.* 2010a). The micropropagated hardened plantlets after confirmation of genetic fidelity, being transferred to experimental garden soil and maintained as a field gene bank, showed robust growth and seed set without any marked phenotypic variations in vegetative or floral characteristics (Bhowmik *et al.* 2009, 2010a,b). The survival rate of these reintroduced plantlets of both the species was almost 100 % in the new environment of Shillong. This might possibly due to more favorable environmental conditions such as acidic pH, lower altitude accompanied by higher rainfall as compared to its native habitat. Finally, re-establishment of the tissue cultured plantlets to their natural habitat (Lunglei, Mizoram) leading to *in situ* conservation of *M. spathulata* and *M. wengeri* has been successfully accomplished to circumvent the risk of extinction of the genus *Mantisia*.

References

Agrawal, D.C., Pawar. S.S., Morwal, G.C., Mascarenhas, A.F.: I*n vitro* micropropagation of *Delphinum malabaricum* (Huth) Munz – a rare species. - Ann. Bot. **68**: 243-245, 1991.

Archibald, J.K., Wolf, P.G., Tepedino, V.J., Bair J.: Genetic relationships and population structure of the endangered steamboat buckwheat, *Eriogonum ovalifolium* var. *williamsiae* (*Polygonaceae*). - Amer. J. Bot. **88**: 608-615, 2001.

Ayres, D.R., Ryan, F.J.: Genetic diversity and structure of the narrow endemic *Wyethia reticulata* and its congener *W. bolanderi* (*Asteraceae*) using RAPD and allozyme techniques. - Amer. J. Bot. **86**: 344-353, 1999.

Beharav, A., Maras, M., Kitner, M., Šuštar-Vozlič, J., Sun, G.L., Doležalová, I., Lebeda, A., Meglič, V.: Comparison of three genetic similarity coefficients based on dominant markers from predominantly self-pollinating species. - Biol. Plant. **54**: 54-60, 2010.

Bhattachary, E., Dandin, S.B., Ranade, S.A.: Single primer amplification methods reveal exotic and indigenous mulberry varieties are similarly diverse. - J. Biosci. **30**: 669-677, 2005.

Bhattacharya, S., Bandopadhyay, T.K., Ghosh, P.D.: Efficiency of RAPD and ISSR markers in assessment of molecular diversity in elite germplasms of *Cymbopogon winterianus* across West Bengal, India. - Emir. J. Food Agr. **22**: 13-24, 2010.

Bhowmik, S.S.D., Kumaria, S., Rao, S.R., Tandon, P.: High frequency plantlet regeneration from rhizomatous buds in *Mantisia spathulata* Schult. and *Mantisia wengeri* Fischer and analysis of genetic uniformity using RAPD markers. - Ind. J. exp. Biol. **47**: 140-146, 2009.

Bhowmik, S.D.S., Kumaria, S., Tandon, P.: Conservation of *Mantisia spathulata* Schult. and *Mantisia wengeri* Fischer, two critically endangered and endemic zingibers of Northeast India. - Seed Technol. Notes **32**: 57-62, 2010a.

Bhowmik, S.D.S., Kumaria, S., Tandon, P.: Rhizome derived callogenic plantlet production of *Mantisia wengeri* (*Zingiberaceae*), a rare and endemic medicinal plant of Mizoram, North-East India. - Int. Res. J. Biotechnol. **1**: 65-70, 2010b.

Dam, D.P., Dam, N., Dutta, R.M.: *Mantisia saltatoria* Sims *versus Globba radicalis* Roxb. (*Zingiberaceae*). - Bull. Bot. Surv. India **34**: 188-193, 1997.

Ellstrand, N.C., Elam, D.R.: Population genetic consequences of small population size: implications for plant conservation. - Annu. Rev. Ecol. Syst. **24**: 217-242, 1993.

Frankham, R.: Conservation genetics. - Annu. Rev. Genet. **29**: 305-327, 1995.

Ganeshaiah, K.N.: Recovery of endangered and threatened species: developing a national priority list of plants and insects. - Curr. Sci. **89**: 599-600, 2005.

Gupta, M., Chyi, Y.S, Romero-Severson, J., Owen, J.L.: Amplification of DNA markers from evolutionarily diverse genomes using single primers of SSRs. - Theor. appl. Genet. **89**: 998-1006, 1994.

Heath, D.D., Iwana, G.K., Delvin, R.H.: PCR primed with VNTR core sequences yield species specific patterns and hypervariable probes. – Nucl. Acids Res. **21**: 5782-5785, 1993.

Kang, S.Y., Lee, K.J., Lee G.J., Kim, J.B., Chung, S.J., Song, J.Y., Lee B.M., Kim, D.S.: Development of AFLP and STS markers linked to a waterlogging tolerance in Korean soybean landraces. - Biol. Plant. **54**: 61-68, 2010.

Kress, W.J.L., Prince, M., Hahn, W.J., Zimmer, E.A.: Unraveling the evolutionary radiation of the families of the *Zingiberales* using morphological and molecular evidence. - Syst. Biol. **51**: 926-944, 2001.

Lande, R.: Genetics and demography in biological conservation. - Science **241**: 1455-1460, 1988.

Ma, K.H., Kim, K.H., Dixit, A., Chung, I.M., Gwag, J.G., Kim, T.S., Park, Y.J.: Assessment of genetic diversity and relationships among *Coix lacryma-jobi* accessions using microsatellite markers. - Biol. Plant. **54**: 272-278, 2010.

McKently, A.H., Adams, J.B.: *In vitro* propagation of *Paronychia chartacea*. - Hort. Sci. **29**: 921-930, 1994.

Pei, Z., Gao, J., Chen, Q., Wei, J., Li, Z., Luo, F., Shi, L., Ding, B., Sun, S.: Genetic diversity of elite sweet sorghum

genotypes assessed by SSR markers. - Biol. Plant. **54**: 653-658, 2010.

Rahman, M.A., Yusuf, M.: *Mantisia salarkhanii* Rahman & Yusuf (*Zingiberaceae*) – a new species from Bangladesh. - Saudi. J. Biol. Sci. **9**: 105-110, 2002.

Ranade, S.A., Rana, T.S., Narzary, D.: SPAR profile and genetic diversity amongst pomegranate (*Punica granatum* L.) genotypes. - Physiol. mol. Biol. Plants **15**: 61-70, 2009.

Rathore, T.S., Tandon, P. Shekhawat, N.S.: *In vitro* regeneration of pitcher plant (*Nepenthes khasiana* Hook. f.) – a rare insectivorous plant of India. - J. Plant Physiol. **139**: 246-248, 1991.

Sikdar, B., Bhattacharya, M., Mukherjee, A., Banerjee A., Ghosh, E., Ghosh, B., Roy, S.C.: Genetic diversity in important members of *Cucurbitaceae* using isozyme, RAPD and ISSR markers. - Biol. Plant. **54**: 135-140, 2010.

Sharma, S.K., Kumaria, S., Tandon, P., Rao, S.R.: Single primer amplification reaction (SPAR) reveals inter- and intra-specific natural genetic variation in five species of *Cymbidium* (Orchidaceae). - Gene **483**: 54-62, 2011a.

Sharma, S.K., Kumaria, S., Tandon, P., Rao, S.R.: Synaptic variation derived plausible cytogenetical basis of rarity and endangeredness of endemic *Mantisia spathulata* Schult. – The Nucleus: doi 10.1007/s13237-011-0033-1, 2011b.

Sharma, S.K., Rawat, D., Kumar, S., Kumar, A., Kumaria, S., Rao, S.R.: Single primer amplification reaction (SPAR) reveals intra-specific natural variation in *Prosopis cineraria* (L.) Druce. - Trees **24**: 855-864, 2010.

Sudha, C.G., Seeni, S.: *In vitro* propagation of *Rauwolfia micrantha*, a rare medicinal plant. - Plant Cell Tissue Organ Cult. **44**: 243-248, 1996.

Takano, A., Okada, H.: Taxonomy of *Globba* (*Zingiberaceae*) in Sumatra, Indonesia. - Syst. Bot. **28**: 524-546, 2003.

Tandon, P., Bhowmik, S.S.D., Mao, A.A., Kumaria, S.: Rapid *in vitro* clonal propagation of *Mantisia spathulata* Schult, a rare and endemic plant of Northeastern India for recovery. - Biotechnology **6**: 68-71, 2007.

Viana, A.J.C., Souza, M.M., Araújo, I.S., Corrêa, R.X., Ahnert, D.: Genetic diversity in *Passiflora* species determined by morphological and molecular characteristics. - Biol. Plant. **54**: 535-538, 2010.

Welsh, J., McClelland, M.: Fingerprinting genomes using PCR with arbitrary primers. - Nucl. Acids Res. **18**: 7213-7218, 1990.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V.: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. – Nucl. Acids Res. **18**: 6531-6535, 1990.

Xue, D.W., Ge, X.J., Hao, G., Zhang, C.Q.: High genetic diversity in a rare, narrowly endemic primrose species: *Primula interjacens* by ISSR analysis. - Acta bot. sin. **46**: 1163-1169, 2004.