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Genetic diversity and population structure assessment of *Malaxis acuminata* D. Don, a threatened terrestrial medicinal orchid using SPAR methods

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Abstract

Malaxis acuminata, is an endangered, terrestrial orchid species endemic to the tropical Himalayas. It is highly valued for its medicinal properties and is commercially used in the preparation of an Ayurvedic tonic 'Chyavanprash'. of late, the plant has been widely exploited from its natural habitat rendering it threatened in nature. Considering its current status, a study was conducted to evaluate the genetic diversity and population structure in order to provide a baseline empirical framework through which conservation practitioners can formulate and ameliorate conservation plans. Genetic variation and population structure among 72 genotypes of *M. acuminata* representing 12 populations from Meghalaya was analysed using four different single primer amplification reaction (SPAR) methods viz., Intron-exon splice junction (ISJ), Directed amplification of minisatellite DNA (DAMD), Inter simple sequence repeats (ISSRs) and Start Codon Targeted polymorphism (SCoT). A totally of 344 amplicons were generated, out of which 281 fragments were polymorphic (81.68 %). Nei's gene diversity (h= 0.1342) and Shannon's information index (I=0.2696) revealed high genetic diversity at intra-population level. Furthermore, high gene flow (N_m =1.440), low genetic differentiation (Gst = 0.170) and high genetic variation (91.19%) within populations were recorded. Bayesian-based structure, neighbour joining (NJ) and Principal Coordinate Analysis (PCoA) two genetically discrete gene pools with overlapping and intermixing of populations. The present investigation demonstrates the efficiency of SPAR technique to assess the genetic diversity and population structure of *M. acuminate* which is a rudimentary point to assist conservation and management programmes of the prevalent spectrum of genetic diversity for this terrestrial orchid.

Keywords : Threatened, *Malaxis acuminata*, Chyavanprash, SPAR, genetic diversity, and population structure

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Introduction

The remedies in the fight against newer and ever threatening diseases lies not with chemicals alone, perhaps, medicinal plants can well be the panacea of the future. Plants have always been the backbone of ancient traditional medicinal wisdom. As one of the most diverse and largest families of flowering plants containing more than 25,000 species (Dressler, 1993), the family Orchidaceae provides a myriad of both known and untapped medical resources. Known for their demand in the cut flower industry, orchids are also highly valued for their medicinal properties.

Not recognized for its ornamental beauty, *Malaxis acuminate* boasts of rich medicinal property and is widely used in traditional system of medicine since the Vedic period (Sharma *et al.,* 2011). It is a small terrestrial orchid commonly known as 'Jeevaka' and is endemic to the tropical Himalayas (Fig.-1). The dried pseudobulbs are an imperative ingredient of 'Ashta-varga' drugs used in the preparation of an ayurvedic

medicine 'Chyavanprash' (Kaur and Bhutani, 2010). The species is also known to possess cardioprotective, adaptogenic, antioxidant, and anti-aging properties. Other documented medicinal attributes include its uses as an immunomodulator, rejuvenator, brain tonic and health promoter (Sharma *et al.,* 2011; Balkrishna *et al.,* 2012). It is also reported that *M. acuminata* is used to cure tuberculosis and is a great aphrodisiac (Chauhan, 1990). Anthropogenic interferences such as indiscriminate land use, jhum cultivation results in rapid destruction of forests, affecting the natural distribution of the species. At the same time, over exploitation of the plant for its pseudobulbs has rendered the plant threatened in nature. *M. acuminate* has been identified as Vulnerable in Western Himalayas by Conservation Assessment and Management Plan (CAMP)-WWF, India and also included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II (Lohani *et al.,* 2013). Hence, a thorough understanding of the availability and distribution of all natural genetic variations is essential to design efficient and sustainable conservation strategies in order to safeguard the remaining wild populations of *M. acuminata.*

The use of molecular analysis as an integral component in the conservation of rare and endangered species has become more widely adopted (Luan *et al.,* 2006). There are various DNA markers used for genetic evaluation and molecular characterization of plants. Intron-exon splice junction (ISJ) markers for instance, emerged to be an alternative to Random Amplified Polymorphic DNA (RAPD) and other tedious and expensive methods such as Restriction Fragment Length Polymorphism (RFLP) and Amplified Fragment Length Polymorphism (AFLP) (Gawel and Iwona, 2002). They are semi-specific markers, based on sequences commonly found in plants and indispensable for post-transcription DNA processing (Weining and Langridge, 1991). ISJ marker is relatively dominant and the amplified sequences are highly conserved (Sawicki and Szczecinska, 2007). Inter simple sequence repeats (ISSRs) (Weber, 1990; Zietkiewiczet *et al.,* 1994) and Directed amplification of minisatellite DNA (DAMD) (Heath *et al.,* 1993) markers have also been extensively used to study population genetics as they are highly polymorphic, reproducible, stable, consistent and dominant making them particularly suitable for evaluating genetic diversity and phylogeny among plant species and populations. Of late, Start Codon Targeted (SCoT) polymorphism emerged as an effective marker technique for cataloguing and evaluating genetic variability in plants (Collard and Mackill, 2009; Bhattacharyya *et al.,* 2013). This technique is sensitive to low levels of genetic variations and thus provides a very useful tool for analysing population genetics on a wide range of plants as well as identifying species or population of the same species (Collard and Mackill, 2009).

The state of Meghalaya, India, is endowed with rich and diversified flora. About 532 species of orchids have been reported so far (Medhi and Chakrabarti, 2009). Orchids in the state are facing serious threats with the increase in biotic influences and unrestrained commercial exploitation of forest wealth. So far, no consolidated publication on the genetic diversity and population structure of *M. acuminata* is available, hence, the present study is aimed at exploring the genetic diversity within and among the wild populations of *M. Acuminate* collected from different parts of Meghalaya. Four Single Primer Amplification Reaction (SPAR) methods using ISJ, DAMD, ISSR and ScoT markers have been employed to understand the genetic variation and population structure in order to formulate strategies for species management and conservation.

Materials and Methods

Study sites and sample collection

Seventy-two plants of *M. acuminata* representing twelve natural populations of Meghalaya (Table - 1) were collected and maintained in the green house of the Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong, Meghalaya, India. Due to the rarity of the number of individuals in each population, the sampling size was determined in such a way so as not to destroy the natural distribution of the plant in the wild. The sampling strategy also ensured the full geographical range of *M. acuminatain* Meghalaya.

Genomic DNA extraction

Total genomic DNA was extracted from the young leaves of *M. acuminata* following the CTAB method with some minor modifications (Doyle and Doyle, 1987). Integrity and quantity of the isolated DNA were evaluated using a UV Spectrophotometer (Perkin Elmer Lambda 35). The ratio of absorbance at two wavelengths (A_{260} and A_{280}) was compared with the standard ratio of pure DNA.

PCR optimization and amplification reactions with ISJ, DAMD, ISSR and SCoT primers

Optimization of the PCR amplification conditions was undertaken with varying concentrations of DNA template (20 - 60 ng), Taq DNA polymerase (0.5 - 2 U), and Mg^{++} salt (1 - 5 mM). After establishing the optimal conditions, amplification reactions of all the genotypes and screened primers were carried out. The PCR amplifications for all the primers contained 50 ng of template DNA, 2 µM of each of the four dNTPs, 1× PCR buffer (10 mM Tris, pH 9.0, 50 mMKCl), 1.5 mM $MgCl_2$, 1 U Taq polymerase (Bangalore Genei, India) and 20 pmol of primer in a reaction mixture of 25 µL volume.

ISJ- PCR Amplification

Thirty eight ISJ primers synthesized by Metabion Inc. Ltd., Germany were primarily screened for optimal amplification. Eventually, 9 primers with reproducible, clearly separated bands and polymorphic patterns were selected for final amplification. The reactions for the amplification of the DNA were carried out under the following conditions: pre-denaturation at 94°C for 4 min; 45 cycles of 45 s at 94°C for denaturation, 50 s at a temperature range of 50 °C-58 °C for primer annealing and 90 s at 72°C for extension, with a final extension for 7 min at 72°C in a thermal cycler 2720 (Applied Biosystems, USA).

DAMD-PCR Amplification

Out of the 20 DAMD primers, 11 primers were screened out for further analysis. PCR was performed at initial denaturation of 94°C for 4 min; 35 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min followed by a final extension at 72°C for 7 min.

ISSR - PCR Amplification

A total of 36 ISSR primers were screened, out of which 10 primers were found suitable for further analysis. The reaction programs were set at 94°C for 5 min, subsequently followed by 40 cycles of 1 min at 94°C, 1 min at an annealing temperature range of 48°C to 58°C depending on the melting temperature of the ISSR primers used and 2 min at 72°C, with a final extension at 72°C for 5 min in a thermal cycler.

SCoT - PCR Amplification

Twelve out of 36 SCoT primers were selected for amplifying the various genotypes. PCR reactions were programmed at 94°C for 3 min, 40 cycles of 1 min at 94°C, 1 min at 50°C, 4 min at 72°C and 72°C for 5 min.

Gel electrophoresis

Amplification products were separated by electro-

Table - 1. Details of populations and geographical locations of *M. acuminata* in Meghalaya

Population Site	District	No. of Samples	Collection Name	Latitude (N)	Longitude (E)	Altitude (m)
Lower Darengre	West Garo Hills	6	LD1-LD6	25.48672	90.28275	1154.2
Nongstoin	West Khasi Hills	6	NS1-NS6	25.53022	91.28415	1396.7
Nongbri	West Khasi Hills	6	NB1-NB6	25.55323	91.67368	1746.3
Sohkyllam	Southwest Khasi Hills	6	SK1-SK6	25.38076	91.51212	1593.1
Pomlum	East Khasi Hills	6	PM1-PM6	25.53314	91.81318	1761.2
Nongkrem	East Khasi Hills	6	NK1-NK6	25.49346	91.87395	1820.0
Pynursla	East Khasi Hills	6	PY1-PY6	25.30477	91.90795	1313.8
LaitMansang	East Khasi Hills	6	LM1-LM6	25.40491	92.02298	1349.5
Umiam	Ri Bhoi	6	UM1-UM6	25.67761	91.90271	1007.9
Nongjri	Ri Bhoi	6	NJ1-NJ6	25.72078	91.92454	1077.6
Jarain	West Jaintia Hills	6	JR1-JR6	25.39034	92.14917	1347.0
Ialong	East Jaintia Hills	6	IL1-IL6	25.45329	92.25051	1338.7

phoresis in 1.5% agarose gel in 1X TAE buffer at 65-70V for 2 hours. DNA fragments were visualized under a UV light and photographed using Gel Documentation System (Biostep DH-20, Germany).

Data scoring and statistical analysis

ISJ, DAMD, ISSR and SCoT marker banding profiles were scored for 72 genotypes of *M. acuminata*. Only clear, distinguishable, and reproducible bands were considered for scoring. The presence (1) and absence (0) of bands were recorded to generate a binary data matrix and to further evaluate the polymorphic loci. To evaluate the discriminatory power of the primers, two parameters were considered i.e. Polymorphic Information Content (PIC) and Resolving Power (Rp). PIC value was calculated in accordance to Smith *et al.* (1997) as: PIC = $1-\sum pi^2$ where pi is the frequency of i^{th} allele (band present) whereas the resolving power (Rp) of each primer was estimated following the formula Rp = ΣIb where band informativeness, $I_b = 1 - (2 \times |0.5 - p|)$ and p is the proportion of genotypes containing band I (Prevost and Wilkinson, 1999). POPGENE version 1.31 (Yeh *et al.*, 1999) was used to estimate genetic diversity parameters including the percentage of polymorphic loci (Pp) (Lynch and Milligan, 1994), Nei's gene diversity (h) (Nei, 1973), Shannon's information index (I) (Lewontin, 1972) and Gene flow ($Nm = 0.25 \times (1 - Gst) / Gst$). The genetic variation among and within populations was also investigated with Analysis of Molecular Variance (AMOVA) using Arlequin version 3.01 (Excoffier *et al.*, 2005). The Fixation index or F statistics (FST) was determined using Arlequin v. 3.01 and the significance of this analog was evaluated by 1000 random permutations of sequences among populations (Miller, 1998).

To gain insights into the genetic structure among the populations, Bayesian model based clustering using structure version 2.3.4 was used (Pritchard *et al.*, 2000; Falush *et al.*, 2007). K value ranging from 1 to 20 with 3 iterations for each value of K was set with length of burn-in period and number of Markov chain Monte Carlo (MCMC) at 100,000 each. Online programme 'Structure Harvester' (Evanno *et al.*, 2005; Earl, *et al.*, 2010) was used to determine the value of K which is best-fit to interpret the present data. Pair-wise distances between genotypes was computed using weighted

Neighbour joining (NJ) method and tree construction was carried out using Darwin version 5.5.158 (Dissimilarity Analysis and Representation for Windows) software (Perrier and Jacquemoud-Collet, 2006). Additionally, Principal Coordinate Analysis (PCoA) was conducted to correlate genetic relationships between the populations using PAST version 3.16 (Hammer *et al.*, 2001).

Results

DAMD analysis

Evaluation of the genetic diversity of 72 samples of *M. acuminate* representing 12 populations was carried out using 11 DAMD primers which generated 98 amplicons, out of which 71 were found to be polymorphic (Table 2 and 3). Size of the amplified products ranged from 0.1 kb to 2.0 kb depending on the primer used and the average number of amplicons per primer was observed to be 8.80 (Fig.-1A). Highest level of polymorphism was exhibited by the primer Oligo-1 (Table - 2).

ISJ analysis

A total of 9 ISJ primers exhibited 70 amplicons, out of which 55 were polymorphic (Table 2 and 3). The average number of amplicons per primer was 7.77 with sizes ranging from 0.2 kb to 1.0 kb (Fig.-1B). Polymorphic bands ranging from 57.14 to 100 % were yielded with primer ISJ7 and IT33 exhibiting the highest level of polymorphism (Table - 2).

Table -2. Data of DAMD, ISJ, ISSR and SCoT primers used in the present study and the extent of polymorphism

Sl. No.	Name of primer	Primer's sequence (5'-3')	Total number of bands	No. of Poly-morphic bands	No. of Mono-morphic bands	% of Poly-morphism	Resolving power (*Rp*)	*PIC**
DAMD								
1	HBV	GGTGTAGAGAGGGGT	9	8	1	88.88	7.55	0.72
2	HBV3	GGTGAAGC CAGGTG	8	5	3	62.50	3.77	0.42
3	HVA	AGGATGGAAAGGAGGC	10	9	1	90.00	6.58	0.59
4	M13	GAGGGTGGCGGTTCT	10	8	2	80.00	7.39	0.51
5	Oligo-1	GGAGGTTTTCA	13	12	1	92.30	8.05	0.82
6	Oligo-4	CCCGTGGGGCCGCCG	6	4	2	66.66	3.97	0.37
7	URP1F	ATCCAAGGTCCGAGA CAACC	12	9	3	75.00	3.77	0.42
8	URP2F	GTGTGCGATCAGTTGCTGGG	8	5	3	62.50	3.05	0.32
9	URP4R	AGGACTCGATAACAGGCTCC	6	3	3	50.00	3.75	0.43
10	URP6R	GGCAAGCTGGTGGGAGGTAC	7	3	4	42.85	4.61	0.41
11	URP13R	TACACGTCTCGATCTACAGG	9	5	4	55.55	3.69	0.39
ISJ								
1	ISJ3	TGCAGGTCAG	8	5	3	62.50	5.66	0.53
2	ISJ5	CAGGGTCCCACCTGCA	6	4	2	66.66	0.72	0.10
3	ISJ7	TGCAGGTCAGGACCCT	9	9	0	100.00	8.25	0.79
4	ISJ9	AGGTGACCGACCTGCA	6	5	1	83.33	2.33	0.27
5	IT3'	GTGCGGCCACAGGTAAGT	9	8	1	88.88	8.66	0.81
6	IT33	GATGCCCCAGGTAAG	9	9	0	100.00	8.25	0.79
7	IT36	ACCTACCGTGGGCTC	7	4	3	57.14	4.05	0.42
8	ET32	ACTTACCTGGGCACG	9	7	2	77.77	4.22	0.43
9	ET33	ACTTACCTGGCCGTG	7	4	3	57.14	4.05	0.42

Sl. No.	Name of primer	Primer's sequence (5'-3')	Total number of bands	No. of Poly-morphic bands	No. of Mono-morphic bands	% of Poly-morphism	Resolving power (*Rp*)	*PIC**
ISSR								
1	B	GAGAGAGAGAGAGAGAT	8	8	0	100.00	7.47	0.75
2	C	ACACACACACACAC CT	6	6	0	100.00	4.13	0.47
3	D	TGTGTGTGTGTGTGTGA	11	11	0	100.00	8.58	0.87
4	G	CCGCCGCCGCCGCCGCCG	5	4	1	80.00	2.08	0.24
5	I	AGAGTTGGTAGCTCTTGATC	9	8	1	88.88	8.66	0.81
6	K	GGCGGCGGCGGCGGCGGC	8	5	3	62.50	1.61	0.21
7	L	CATGGTGTTCATCATTGTTCCA	10	9	1	90.00	7.41	0.74
8	N	CACACACACACAGG	9	7	2	77.77	5.47	0.59
9	O	GTGTGTGTGTGTGG	6	4	2	66.66	1.61	0.18
10	R	CACACACACACAGT	11	11	0	100.00	11.25	1.10
SCoT								
1	S2	CAACAATGGCTACCACCC	9	9	0	100.00	7.38	0.76
2	S3	CAACAATGGCTACCACCG	8	7	1	87.50	6.11	0.61
3	S6	CAACAATGGCTACCACGC	7	7	0	100.00	1.83	0.23
4	S9	CAACAATGGCTACCAGCA	7	5	2	71.42	2.44	0.27
5	S14	ACGACATGGCGACCACGC	7	6	1	85.71	4.86	0.48
6	S18	ACCATGGCTACCACCGCC	5	5	0	100.00	2.38	0.27
7	S22	AACCATGGCTACCACCAC	8	8	0	100.00	7.72	0.72
8	S26	ACCATGGCTACCACCGTC	6	6	0	100.00	5.72	0.57
9	S27	ACCATGGCTACCACCGTG	12	11	1	91.66	10.25	1.05
10	S30	CCATGGCTACCACCGGCG	7	6	1	85.71	5.61	0.60
11	S31	CCATGGCTACCACCGCCT	10	8	2	80.00	9.55	0.92
12	S34	ACCATGGCTACCACCGCA	7	4	3	57.14	2.58	0.28

Table – 3. Comparison of SPAR methods (DAMD. ISJ, ISSR and SCoT), individually and collectively.

Sl. No.	Name of SPAR approach	No. of primer used	Total bands amplified	Average bands/ primer	Size of amplicons (range kb)	Total no. of Polymorphic bands	% of Poly-morphism
1	DAMD	11	98	8.90	0.1-2.0	71	72.44
2	ISJ	9	70	7.77	0.2-1.0	55	78.57
3	ISSR	10	83	8.30	0.1-1.0	74	87.95
4	SCoT	12	93	7.75	0.1-2.0	82	88.17
5	DAMD+ISJ+ISSR+SCoT	42	344	8.20	0.1-2.0	281	81.68

ISSR analysis

Ten ISSR primers were selected for evaluating the genetic diversity of *M. acuminata*. A total of 83 amplification products were scored, yielding an average of 86.58% polymorphism (Table 2 and 3). The average number of amplicons was 8.30, with primer D and R showing the highest number of bands (11 bands) and the highest level of polymorphism. Size of the amplified products ranged from 0.1 kb to 1.0 kb (Fig.- 1C).

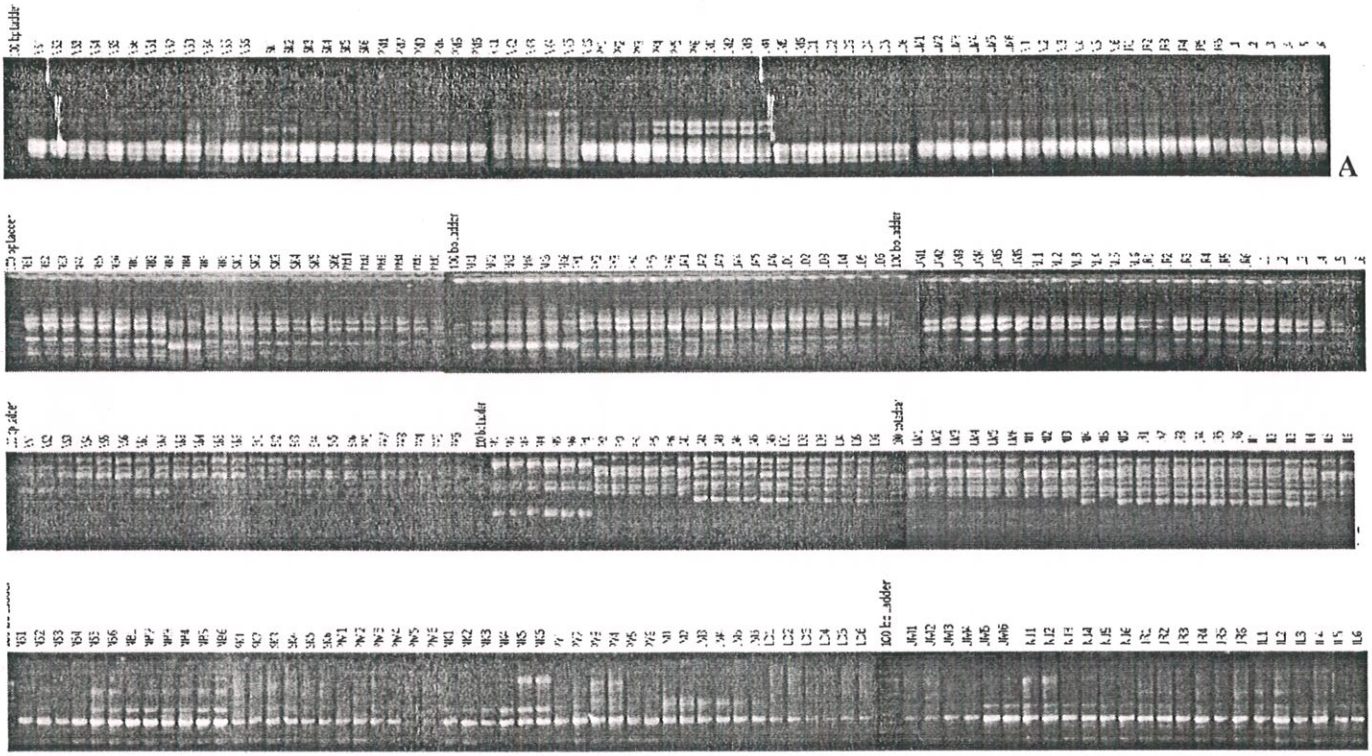

Fig. - 1. Banding profile in *M. acuminata* populations using DAMD primer M13 (A) ISJ primer IT36 (B) ISSR primer B (C) and SCoT primer S27 (D)

SCoT analysis

Twelve SCoT primers generated a total of 93 amplicons with about 7.75 amplicons per primer out of which 82 bands were polymorphic (Table 2 and 3). Polymorphism exhibited by primers across the accessions ranged from 57.14% (S34) to 100 % (S2, S6, S18, S22, S26) with an average of 88.26% poly-morphism. Size of the amplified products ranged from 0.1 kb to 2.0 kb (Fig.-1D).

Genetic diversity

In the present investigation, four SPAR methods viz. DAMD, ISJ, ISSR and SCoT, were used in combinatorial approach, and were seen to exhibit distinct banding patterns for all the primers across all the accessions studied. The percentage of polymorphic loci (Pp) of the combined data ranged from 32.55% to 75.44%. Nei's gene diversity (h) and Shannon's information index (I), varied from 0.0431 ± 0.1650 to 0.2031 ± 0.2736 (0.1342 ± 0.2340) and 0.0306 ± 0.1175 to 0.1351 ± 0.1910 (0.2696 ± 0.1632), respectively. Inter-population genetic diversity (Gst) calculated was 0.170 while at intra-population level (Hpop), genetic diversity was found to be 0.297 (Table - 4).

Population structure

AMOVA analysis was carried out to estimate population differentiation among accessions of *M. acuminata*. High variation (91.19 %) was revealed within the population as compared to low variation between the populations (8.81 %) (Table - 5). The overall Fixation index or F statistics (F_{st}) (0.088) indicates low genetic differentiation among the populations studied. Gene flow (N_m) was calculated using Wright's equation (1951) and was measured to be 1.440 (Table - 4).

Table - 4. Genetic diversity and differentiation parameters for twelve wild populations of *M. acuminatain* Meghalaya

Population	*Ss*	*Na* ± SD	*Ne*± SD	*h*± SD	*I*± SD	*Pp* (%)	*Hsp*	*Hpop*	*Gst*	*Nm*	F_{ST}
Lower Darengre	6	1.2043±0.4054	1.1247±0.2798	0.1095±0.2259	0.0732±0.1547	38.08					
Nongstoin	6	1.3118±0.4658	1.1968±0.3450	0.1689±0.2625	0.1130±0.1801	53.19					
Nongbri	6	1.2581±0.4399	1.1636±0.3046	0.1465±0.2477	0.0972±0.1693	45.19					
Sohkyllam	6	1.3678±0.3221	1.2243±0.3443	0.1951±0.2750	0.1311±0.1895	70.46					
Pomlum	6	1.2796±0.4512	1.1671±0.2927	0.1665±0.2325	0.1045±0.1583	48.25					
Nongkrem	6	1.3978±0.4921	1.2343±0.3607	0.2031±0.2736	0.1351±0.1910	75.44					
Pynursla	6	1.2903±0.4564	1.1920±0.3251	0.1667±0.2651	0.1122±0.1846	51.74					
Lait Mansang	6	1.3548±0.4811	1.2174±0.3668	0.1747±0.2803	0.1205±0.1961	55.52					
Umiam	6	1.0645±0.2470	1.0559±0.1882	0.0431±0.1650	0.0306±0.1175	32.55					
Nongjri	6	1.2301±0.4138	1.1351±0.2829	0.1241±0.2283	0.0810±0.1559	40.69					
Jarain	6	1.0968±0.2973	1.0587±0.2258	0.0513±0.1617	0.0339±0.1090	35.46					
Ialong	6	1.1460±0.3134	1.0809±0.2545	0.0620±0.1914	0.0435±0.1350	36.04					
Total		1.3453±0.4159	1.2442±0.3875	0.1342±0.2340	0.2696±0.1632	61.68	0.300	0.297	0.170	1.440	0.088

Ss = No. of individuals; *Na* = Observed number of alleles; Ne = Effective number of alleles; *h* = Nei's gene diversity; *I* = Shannon's information index; *Pp* = Percentage of polymorphic loci; *Hsp* = Total variability; *Hpop* = Variability within population; *Gst* = Diversity among populations, N_m = Gene flow 0.25(1-*Gst*)/*Gst*; F_{ST} = Fixation index or F statistics. SD = Standard deviation.

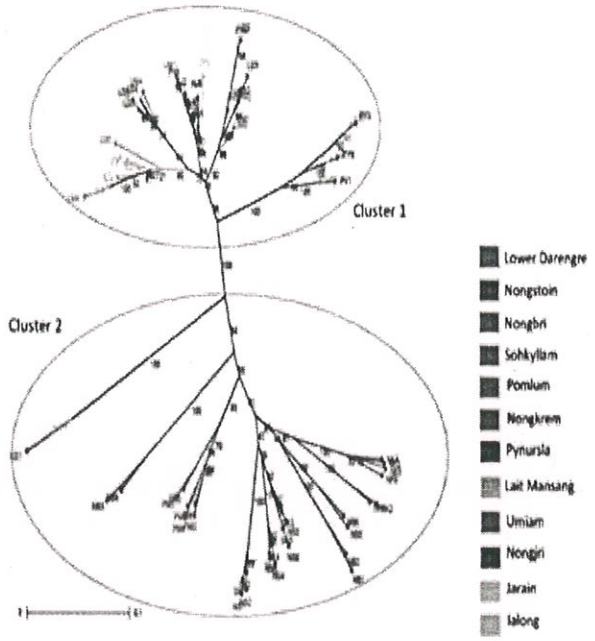

Fig.-2. Bootstrapped neighbour-joining (NJ) tree of 72 *M. acuminata* accessions.

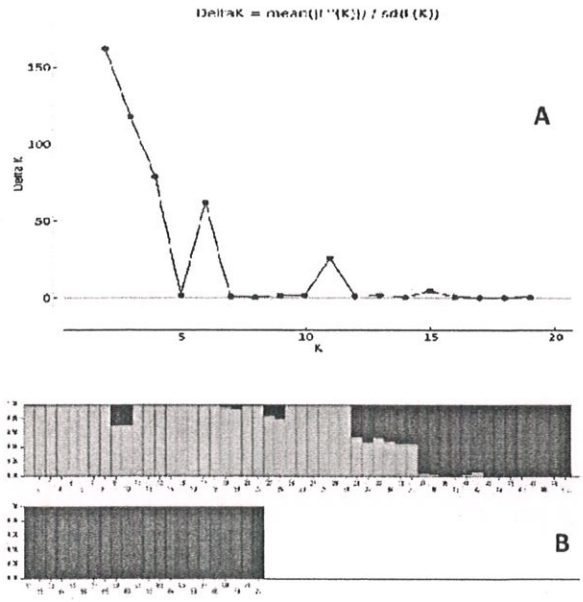

Fig.- 3. Structure analysis based clustering of 72 individuals of *M. acuminate* (A) Plot showing the ΔK values (B) Genetic clustering estimated (K=2) showing two genetic pools.

Table - 5. Results of analysis of molecular variance (AMOVA) for twelve populations of *M. acuminate*

Source of variation	Degrees of freedom (d.f.)	Variance component	Sum of squares	Percentage of variation (%)
Among populations	11	7.917	0.04402 Va	8.81
Within populations	60	27.333	0.45556 Vb	91.19

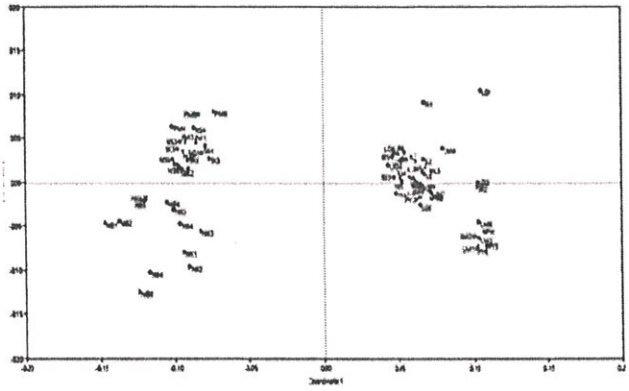

Fig.-5. Principal Coordinate Analysis (PCoA) revealing the clustering pattern of 72 accessions of *M. acuminate*

Based on the NJ tree constructed, the sampled accessions of *M. acuminata* from various places formed two clusters which indicated two significant genetic pools (Fig.-2). There was no clear distinction among the populations as the genotypes were intermixed between the two clusters. The findings corroborated with AMOVA results were higher degree of genetic variation existed within the populations as compared to that recorded among the populations. Bayesian clustering using structure analysis generated congruent results and the populations were grouped into two clusters where the optimal ΔK for K = 2 is the best fit model (Fig.- 3A, B). Furthermore, PCoA result (Fig.-4) was consistent with that revealed by the structure program with significant intermixing of individuals from different populations which suggested weak differentiation as there is a continuous exchange of genes among the populations.

Discussion

In spite of the monumental medicinal importance of *M. acuminata,* there is no documented information on the genetic diversity of the plant. At large, they are widely exploited for their ethnobotanical importance rendering them vulnerable in nature. Knowledge of genetic variation is fundamental to designing strategies for conservation, since the primary goal of conservation is to preserve the prevalent spectrum of genetic diversity and thus the evolutionary potential (Holsinger and Gottlieb, 1991). The generation of knowledge about genetic variation and population structure of rare species has become a common prelude to conservation planning (Ellstrand and Elam, 1993). Hence, a challenging and critical need of the hour is to conserve the existing genetic resources by under- standing the pattern of genetic diversity and population structure of the assessed genotypes. Spar approach has been executed to study the genetic diversity within and among populations of several orchids and other plant species (Kumar *et al.,* 2010; Sharma *et al.,* 2010). In the present study, four PCR based Spar methods were compared to examine the existing natural variations in 72 genotypes of *M. acuminata* collected from Meghalaya.

Two parameters i.e., PIC and Rp were used to determine the effectiveness and discriminatory power of primers. The PIC and Rp values for all the four SPARs were significantly high denoting high number of informative bands generated using these markers. Out of the four markers used, SCoT marker yielded

the highest number of polymorphic amplicons (88.17 %) with significant PIC (0.27-1.05) and Rp (1.83 - 10.25) values classifying it as highly informative and is in concordance with the optimal PIC (Smith *et al.,* 1997) and Rp values (Prevost and Wilkinson, 1999). Gene flow and genetic differentiation are important parameters to infer the population genetic structure of a species (Song *et al.,* 2010). High levels of genetic variation existed within populations with significantly less genetic differentiation among the tested populations which is suggested by the value of Gst (0.170). Gene flow (Nm) (1.440) estimated based on Gst implied high gene flow and this level of migration will prevent divergence among populations (Wright, 1951). The above findings were in tandem with the AMOVA results that exhibited maximum variation within the population (91.19%) and was verified by cluster analysis in Bayesian-based structure, NJ and PCoA. Pollination mechanism and seed dispersal coupled with landscape dynamics are known to be directly related with genetic variation and their partitioning within and among populations (Rawat *et al.,* 2017). Orchid seeds being very light and minute results in long dispersal by wind promoting gene flow (Borba and Semir, 2001) accounting for the overlapping and intermixing of individuals from different populations which gradually results in high genetic diversity at intra population level and low genetic variation between populations (Hamrick and Godt, 1996; Hogbin and Peakall, 1999; Zawko *et al.,* 2001; Manners *et al.,* 2013), as seen in the current study and mostout-breeding species.

Maintenance of genetic diversity is of paramount importance for conserving endangered and threatened species (Avice and Hamrick, 1996). It has been regarded as a factor of a species to adapt to changing environment and climatic conditions for long term existence (Rawat *et al.,* 2017). Knowledge of genetic diversity among and within populations yield essential information in the formulation of suitable management strategies directed towards their conservation (Milligan *et al.,* 1994). Current study revealed a good amount of genetic diversity present within the *M. acuminata* accessions. High genetic diversity maintained in threatened and rare plants is attributed to a number of factors, such as recent reduction of population size and insufficient time for isolation, or widespread, persistent gene flow and a wide range of geographic distribution (Hamrick, 1989). Cluster analysis with all four marker systems showed that there is a lack of correlation between genetic distances and geographic distances. Given that the genetic structure of *M. acuminata* showed high gene flow, low population divergence and high genetic diversity, this indicates that genetic drift is currently not of great concern for this species. The vulnerable status of the species can be attributed to anthropogenic and ecological factors such as habitat destruction, over-exploitation leading to the decline and scattering of such populations.

Conclusion

Conclusively, the present assessment using four Spar methods inferred the genetic diversity and population structure in selected populations of *M. acuminata.* High genetic diversity within populations suggests protection of all extant natural populations in order to sustain the existing germplasm bank. The documentation delivered preliminary insights in order to implement efficient conservation strategies to ensure long-term survival of this threatened medicinal orchid. Present report is the first on analysis of genetic diversity and population structure in *M. acuminata* by the application of SPAR based molecular markers.

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