

Analysis on Genetic diversity of Threatened Medicinal epiphyte *Pleione maculata* from different Geographical habitats using RAPD and ISSR marker

Pleione maculata is a threatened medicinal epiphyte, as pseudobulbs are used to cure headaches, stomachaches and liver diseases. There are currently no reports on genetic diversity studies of *P. maculata* utilizing a molecular marker system that is relevant for conservation and breeding. Therefore, patterns of genetic variability and intraspecific similarities across *P. maculata* from three different geographical distributions were assessed in this study using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) markers. From the 16 RAPD and 2 ISSR markers evaluated, a total of 96 distinct and reproducible DNA amplicons were generated. The morphological dissimilarity matrix was calculated using the Mantel test and Nei's genetic diversity coefficient. The highest computed PIC value for RAPD was 0.44, whereas the highest PIC values for ISSR were 0.48. The two sets of molecular markers used were highly correlated, with a correlation Mantel statistic (r) of 0.79, indicating high marker efficiency in population genetic studies. The results of this study will provide a basis for understanding genetic link, genetic variation and strategies for establishing a germplasm collection for the species' conservation, management, breeding, and hybrid production within the *P. maculata* population.

Key words: *Pleione maculata*, RAPD, ISSR, Genetic diversity.

Introduction

Orchids constitute various habitats, i.e., terrestrial, lithophytic, or epiphytic in nature. Epiphytic orchid faces extensive habitat loss due to urbanization, and deforestation as they are dependent on host trees for support (Sympli *et al.*, 2021). The genus *Pleione* D. Don orchid (Epidendroideae; Coelogyninae) comprises of ~20-23 species and can be easily recognized from other orchid genera (Gravendeel *et al.*, 2004). *Pleione* species though widely used in the cut-flower industry and traditional medicines, very little is known about its evolutionary relationships (Gravendeel *et al.*, 2004). Two sections of *Pleione* were recognized as *Humiles* and *Pleione* (Zhu and Chen, 1998). *Pleione* orchids are one of the unexplored genera of medicinal orchids (Sympli *et al.*, 2018). *Pleione maculata* is rare, endangered, and listed as threatened in Biological Diversity Act. 2002 (Agrawala and Singh, 2013). *Pleione maculata* is a medicinal epiphyte used in the Northeastern regions of India to treat liver problems, stomach ailments, and headaches (Pant, 2003; Teoh, 2016; Sympli *et al.*, 2021). *Pleione. maculata* has slightly inflated florescence sheaths, oblong lip shape and 5-7 lamellae lip ornamentation (Gravendeel *et al.*, 2004).

Due to the morphological character similarities in orchids, it becomes difficult to identify their specific genus and family, therefore molecular characterization at the DNA level is one alternative measure to eliminate confusion in identification (Ferdiani *et al.*, 2015). Genetic diversity, relationships, and classifications can be easily determined with the help of molecular analysis which prevents various traditional taxonomy conflicts (Jobst *et al.*, 1998; Sesli and Yegenoglu, 2009; Rolim *et al.*, 2011). The conventional phenotypic characters are directly or indirectly influenced by environmental factors. Therefore, it did not provide reliable, efficient, and

A base idea is provided to understand genetic link, genetic variation and strategies is for establishing a germplasm collection for conservation, management, breeding and hybrid production within the *Pleione maculata* population.

**HAKANI D. SYMPLI AND
VEDANT VIKROM BORAH**

Department of Bioscience,
Assam Don Bosco University,
Tapesia Garden, Sonapur, India,
Assam-782402.

Email: symplihakani@gmail.com

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accurate information about the phenotypic (morphological) characters (Sesli and Yegenoglu 2009; Jain *et al.*, 2015). Therefore, DNA-based molecular markers are effective and reliable as DNA is not affected by environmental factors (Ninejad *et al.*, 2009; Haider *et al.*, 2012; Souza Neto *et al.*, 2014). They are most suitable for intra-species genotypic variation study and classification into nominal genotypic categories. PCR-based molecular markers such as RAPD and ISSR markers are being progressively used for phylogenetic studies, germplasm studies, and DNA fingerprinting (Parab *et al.*, 2008; Jain *et al.*, 2015). They exhibit various numbers of polymorphisms and do not show pleiotropy characters (Souza Neto *et al.*, 2014).

Molecular marker such as RAPD (William *et al.*, 1990; Niknejad *et al.*, 2009; Rolim *et al.*, 2011) and ISSR has been used due to their cost-effectiveness, robustness, and simple characteristics, but ISSR markers are more reproducible, polymorphic, and informative, hence used to validate RAPD observations (Tikendra *et al.*, 2019). ISSR markers are more stringent as it requires higher annealing temperature (Kishor and Devi, 2009; Haider *et al.*, 2012; Hartati, 2017). ISSR markers have demonstrated a successful identification in gene diversity and used to determined genetic stability (Kishor and Devi, 2009; Hartati, 2017). ISSR markers can distinguish heterozygote and homozygote (Sreedhar *et al.*, 2007) and can unveil changes in repetitive sequences in genome of plants (Palombi and Damiano, 2002; Tikendra *et al.*, 2019).

An effective strategy for conservation of threatened and endangered species is to estimate their genetic diversity and its distribution pattern (Banu *et al.*, 2015). Orchid species have differences when they are grown in different climatic and geographical conditions (Ari *et al.*, 2005). Due to the splitting morphological characters in pseudobulbs and different geographical temperatures of *Pleione maculata*, a study on its genetic relationship and diversity has to be implemented. *P. maculata* epiphyte is not well-studied in the scientific field and no specific molecular markers develop, hence RAPD and ISSR are more efficient to analyze the genetic diversity in higher plants (Sharma *et al.*, 2011).

Therefore, the objective of study was to analyze the genetic relationship and diversity of *P. maculata* within same species originated from three different geographical regions using two marker systems such as RAPD and ISSR molecular markers, which is more reliable for genetic homogeneity and do not require prior knowledge of the sequence. Knowledge of the genetic

structure and diversity of plants creates a major impact in preservation, cultivation, conservation, and plant improvement studies (Pinheiro *et al.*, 2012). This is the first report on molecular analysis of *P. maculata* using molecular marker systems and comparison within same species obtained from three different geographical regions.

Material and Methods

Plant material collection

The young leaves of an epiphyte *P. maculata* were obtained from three distinct habitats of the Northeastern states as presented in (Table 1). (a) Khliehriat, East Jaintia Hills District, Meghalaya (b) Siroi and Kouburu Hills, Manipur and (c) Roing, lower Dibang Valley, Arunachal Pradesh. The samples were stored at -20°C in a zip-lock plastic bag until DNA extraction.

Genomic DNA extraction

Genomic DNA was extracted using the traditional modified Cetyl-trimethyl-ammonium bromide (CTAB) method by Murray and Thomsom (1980) and HiPer-Plant Genomic DNA Extraction Kit (Column based) method (HiMedia, Product code: HTBM004).

Primer selection

RAPD and ISSR primers were obtained from Eurofins Genomics India Pvt. Ltd, Bangalore, India. A total of 16 RAPD-oligonucleotide and 2 ISSR primers were selected based on their higher polymorphism observed from other related genera of epiphytic orchids. The ISSR primers were performed to confirm and validate results produced by RAPD markers. The master stock primers (100 μM) and working stock (10 μM) were prepared for preliminary screening by diluting master stock 1:10 with molecular grade H_2O .

PCR amplification using RAPD markers

PCR was performed in a 0.2 mL microcentrifuge tube with a total volume of 15 μL reaction mixtures consisting of 2X PCR Taq Mixture (HiMedia, MBT061-100R), 10 μM RAPD primers, 50 ng template DNA and 5% DMSO, and 1.5 μM MgCl_2 . Positive and negative controls were prepared without the genomic DNA. The RAPD-PCR reaction was programmed with initial denaturation of 94°C for 3 minutes followed by 35 cycles at 94°C denaturations for 30 seconds, with annealing temperature varied according to their melting temperature (ranges $34-50^{\circ}\text{C}$) for 1 minute, and 1 minute extension at 74°C . The last cycle was followed by a final extension of 74°C for 5 minutes, and a hold time of 12°C

Table 1 : Origin of *Pleione maculata* samples used in the study

Sl. No.	State of collection of <i>P. maculata</i>	Origin	No. of sample	Temperature range
1	Meghalaya (ML)	Khliehriat, East Jaintia Hills District	1	$15^{\circ}\text{C} - 27^{\circ}\text{C}$
2	Manipur (MN)	Siroi & Kouburu Hills	1	$16^{\circ}\text{C} - 20^{\circ}\text{C}$
3	Arunachal Pradesh (AP)	Roing, lower Dibang Valley	1	$6^{\circ}\text{C} - 32^{\circ}\text{C}$

for 15 minutes.

PCR amplification using ISSR markers

The ISSR-PCR reaction with positive and negative control was programmed with initial denaturation of 95°C for 3 minutes followed by 35 cycles of 95°C denaturation for 30 seconds, annealing temperature of 52°C for 1 minute, and extension of 74°C for 1 minute. The last cycle was followed by a final extension of 74°C for 5 minutes, and a hold time of 12°C for 10 minutes.

The amplification of DNA using both RAPD and ISSR markers was performed in a thermal cycler (BIO-RAD T100). The amplified DNA fragments were added with 2 µL of 10X blue gel loading dye, BIO-RAD, Mini-Sub Cell GT in 1.2 – 1.5% Ultra resolution agarose gel (HiMedia) run in 1X TAE buffer at 100 V, 90 Amp, for 60 minutes. The molecular sizes of amplified products were estimated using a 1 kb DNA ladder (HiMedia, MBT05105). The amplified DNA fragments were visualized in UV-light using BIO-RAD, UVView Mini Transilluminator, 100-240V (Model N0: 166-0531). The reactions of RAPD and ISSR primers were repeated in

duplicates to ensure the reproducibility of the banding pattern.

Data analysis

The samples of an epiphyte *P. maculata* collected from three distinct habitats of the Northeastern states as presented in Table 1. (a) Khliehriat, East Jaintia Hills District, Meghalaya (b) Siroi and Koubru Hills, Manipur and (c) Roing, lower Dibang valley, Arunachal Pradesh were statistically analyzed. The polymorphic information content (PIC) and Heterozygosity (H) values were calculated using Gene-Calculator (https://gene-calculator.pl/pic) provided in genetic tools (Bińkowski and Miks, 2018). Banding profiles generated by RAPD and ISSR were pooled into a binary matrix based on the presence (1) and absence (0) of the selected bands made using NTedit 1.07c. The informative bands were used to calculate genetic diversity based on the Nei72 arithmetic coefficient using the SIMGEN procedure from NTSYSpc 2.02 (Numerical Taxonomy and Multivariate Analysis System, Applied Biostatistics Inc., NY, USA) in the software packages. The resultant similarity matrix was employed to construct a dendrogram using the

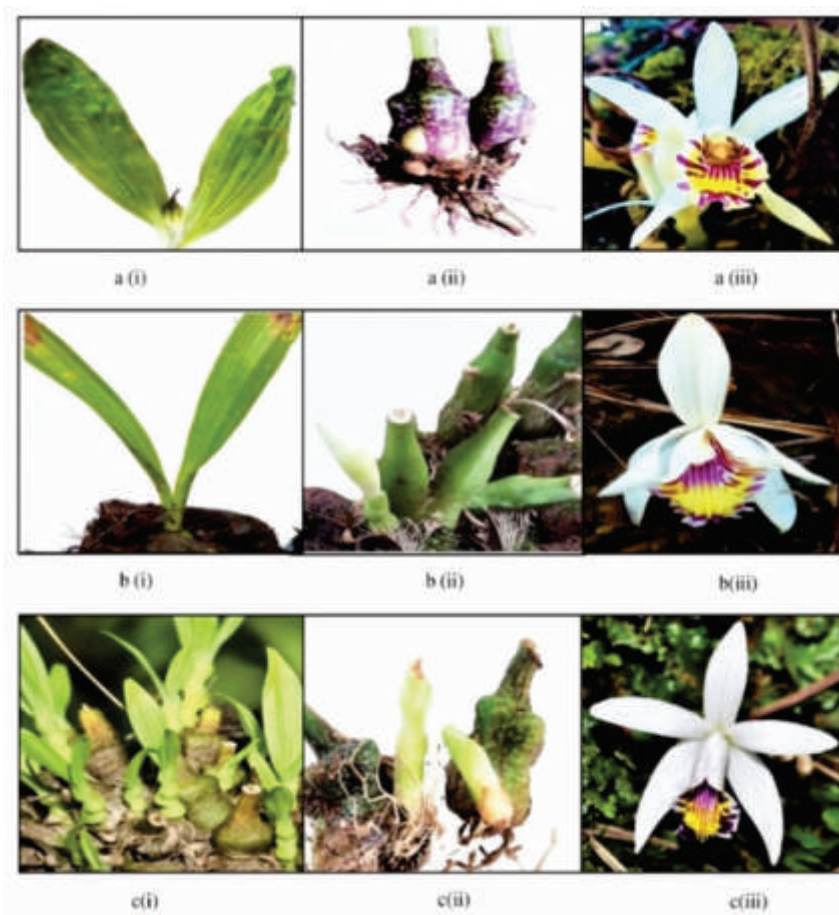


Fig. 1 : The morphotype representation (a) *P. maculata* from Meghalaya (b) *P. maculata* from Manipur and (c) *P. maculata* from Arunachal Pradesh, where (i) leaves (ii) pseudobulb and (iii) flower

unweighted pair group method (UPGMA) cluster algorithm with the module of SAHN procedure from NTSYSpc 2.02 (Hartati, 2017).

A genetic distance and correlation matrices were also calculated using the SIMINT procedure based on DIST coefficient between RAPD and ISSR markers and was compared using MXCOMP procedure. The correlation (r) between the matrices of genetic distance obtained from RAPD and ISSR was tested using the normalized Mantel Z- statistics (Mantel, 1967; Zeng *et al.*, 2004; Tikendra *et al.*, 2019).

Results

Morphological characters

The morphological differences between pseudobulbs of *P. maculata* from three different geographical habitats (Meghalaya, Manipur, and Arunachal Pradesh) are shown in Fig. 1.

Pleione maculata collected from three distinct geographical Northeastern states of India did not represent common morphological characters and their blooming season varies (Table 2) from October to November for Manipur (PM-MN), October to early January for Meghalaya (PM-ML), late October to December in Arunachal Pradesh (PM-AP). The UPGMA dendrogram for morphological study was constructed using NTSYSpc software (Fig. 2) which specifies PM-ML and PM-AP are phylogenetically close and morphologically dissimilar with a rescale distance of 0.22 whereas PM-MN gives a completely another major clade with a rescale distance of 0.57 showing high variation in morphology from PM-ML and PM-AP.

Broad, dark green leaves with prominent parallel venation characterize the morphotype of PM-ML. The pseudobulbs are ovoid, purplish-green in color, and relatively short and stout. The flower are large and showy, with white petals and sepals, and a distinctly fringed labellum that exhibits bold purple striations and a yellow central crest as shown in Fig. 1.

A narrower, lanceolate leaves that are lighter green in color characterized the morphotype of PM-MN. The pseudobulbs are elongated, cylindrical, and brighter green with active growth buds emerging at the base. The flowers are similar in general structure but slightly smaller compared to PM-ML morphotype. The labellum is prominently colored with yellow and purple, though it has less pronounced fringing as shown in Fig. 1.

A clustered growth with multiple upright shoots bearing thin and short leaves characterized the morphotype of PM-AP. The pseudobulbs are more irregular in shape, varying from knobby to slightly bulbous, and pale green to yellowish in color. The flowers are star-like in appearance, with slender white petals and sepals, and a less fringed labellum showing subtler purple and yellow markings as shown in Fig. 1. The three morphotypes shows morphological differences in, leaf shape, pseudobulbs structure, and floral traits.

DNA extraction

DNA extraction with the traditional CTAB method did not provide good quality DNA for PCR-based studies. Pure and high yield of genomic DNA was obtained using the column KIT-based method. The ratio of extracted DNA₂₆₀/A₂₈₀ was 1674 1834 ng/μL.

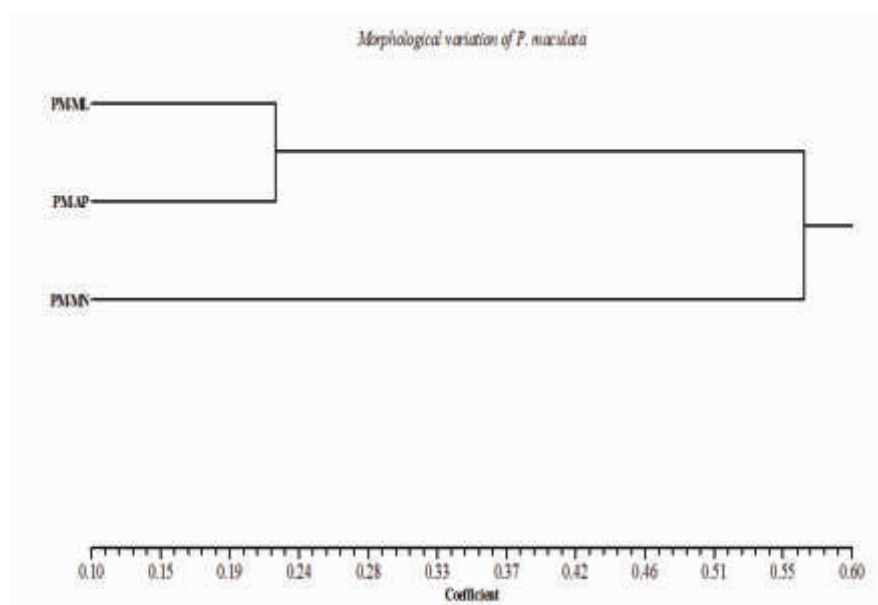


Fig. 2 : UPGMA dendrogram of the morphological characters showing phylogenetic relationship between *P. maculata* from three different geographical regions

Table 2 : The morphological characters of *Pleione maculata* was collected from three different geographical of Northeastern India

<i>Pleione maculata</i> collection states	Morphological characters								
	Plant height (in mm)	No. of leaves	Leaf		Pseudobulb			Flowering period	Flower size (in mm)
			Color	Shaped	Shaped	Colour	Length (in mm)		
Meghalaya (Origin/ Locality- Khliehriat, East Jaintia Hills District)	7-9	2	medium green	Linear folded pleats like	pear- shaped	green with purplish patches	3 - 3.5	October – early January	3-3.2
Manipur (Origin/ Locality- Siroi and Koubru Hills)	6-8	2	dark green	Linear folded and hard pleats like	narrowly ovoid or ovate shaped	completely light green	3.5 - 4	October- November	2.7-3
Arunachal Pradesh (Origin/ Locality- Roing, lower Dibang Valley)	6-8	2	medium green	Linear folded pleats like	pear- shaped	dark green with slight purplish spots	3 – 3.2	October- late December	3.4

Molecular markers

A total of 96 polymorphic and monomorphic DNA amplicons (77 RAPD and 19 ISSR) were generated using 18 primers. A total of 16 dominant RAPD primers were screened and their selection was based on 100% polymorphism, out of which 9 primers gave reproducible bands and showed amplification in *P. maculata* from three states whereas 7 primers did not give any amplification. The total band amplified was 77 out of which 10 bands were polymorphic, while the rest were monomorphic. The number of bands generated by primers varied from 1 (OPD 08 and OPA 12) to 6 bands (RR1).

The size of amplified fragments ranges from 200

–1250 bp (Fig. 3, Table 3). RR1 primer produced a maximum of 6 scorable bands and the lowest number of bands was obtained in OPD 08 and OPA 12 with only one monomorphic band each. The RAPD profile of AP-2-23, RR1, and PER1 primers showed a prominent banding and non-banding pattern indicating slight genetic variation between *P. maculata* from three different geographical regions (Fig. 4). The primer AP-2-23, RR1 and PER1 showed monomorphic banding between PM-ML and PM-AP with 6 scorable bands by RR1, 5 bands by PER1, and 2 bands by AP-A-23. The size of the amplifies band ranges from 225bp to 1250bp.

The AP-2-23 and RR1 showed no bands in PM-MN whereas PER1 showed 3 monomorphic bands with PM-

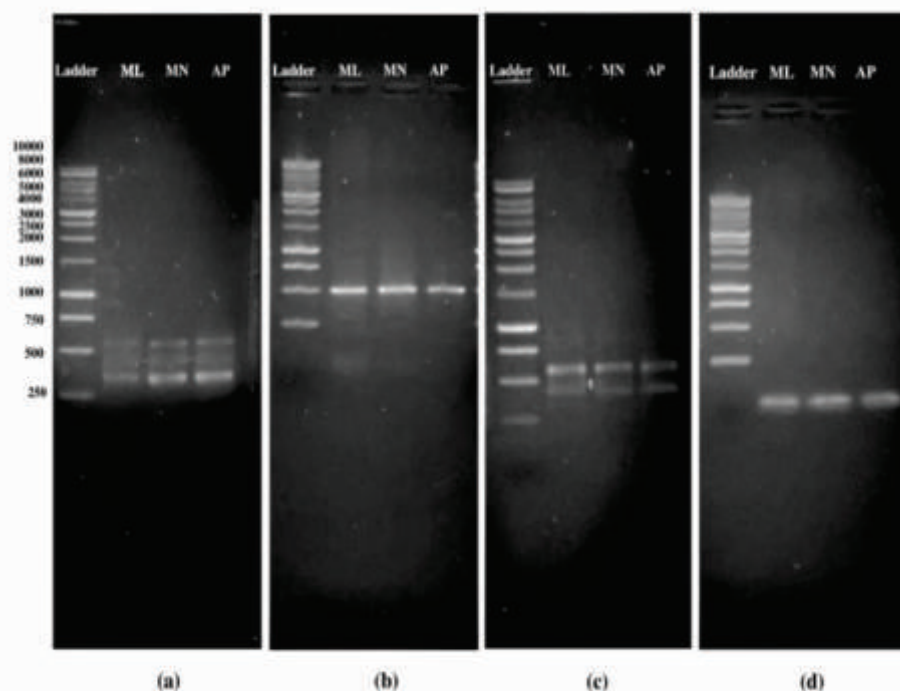
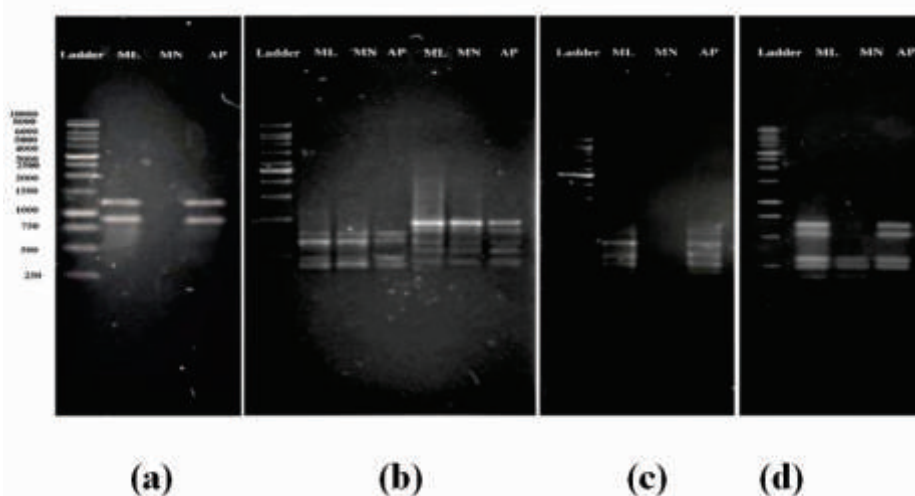


Fig. 3 : PCR- amplicons of three *Pleione maculata* samples by RAPD primers: (a) OPA 11 (b) OPD 08 (c) P2 and (d) OPA 12, whereas, Lane 1- Ladder, lane 2- ML (Meghalaya), lane 3- MN (Manipur), and lane 4- AP (Arunachal Pradesh)

Table 3 : Quantitative data of the PCR- amplicons of *P. maculata* by RAPD primers

RAPD	Primer sequence (5 to 3)	Annealing Tm [°C]	No. of scorable bands	No. of poly-morphic bands	No. of mono-morphic bands	% of poly-morphism (p)	Poly-morphic information content (PIC)	Molecular weight (bp)
OPD 11	AGCGCCATTG	50	9	0	3	0	0.00	275-630
OPD 08	GTGTGCCCA	50	3	0	1	0	0.00	500
P2	GATCGGACGG	38	6	2	2	0	0.00	370-625
OPA 12	TCGGCGATAG	45	3	0	1	0	0.00	200
AP-2-23	GATCTGACTG	35	4	2	2	50	0.44	875-1250
OPA 08	GTGACGTAGG	50	12	0	4	0	0.00	470-750
OPA 13	CAGCACCCAC	50	15	0	5	0	0.00	500-990
CORR1	TGCTCTGCCC	38	12	6	6	50	0.44	250-850
PER1	AAGAGCCCGT	34	13	2	3	15.39	0.23	225-625
Total of bands			77	10	27		1.12	
Average							0.124	


Fig. 4 : PCR- amplicons of three *Pleione maculata* samples by RAPD primers: **(a)** AP-2-23 **(b)** OPA 08 and OPA 13 **(c)** CORR1 and **(d)** PER1, Lane 1- Ladder, lane 2- ML (Meghalaya), lane 3- MN (Manipur), and lane 4- AP (Arunachal Pradesh)

MN and PM-AP at different band ranges 225 bp, 250 bp, and 265 bp. RAPD analysis has highlighted the differences and similarities within the species. The observation of genetic diversity contributes to the molecular genetic relationship and variation within the population of *P. maculata*. AP-A-23 and CORR1 exhibited a 50% degree of polymorphism in PM-MN against PM-ML and PM-AP. A total of two ISSR primers were screened, in which both the primers generated reproducible bands. UBC 810 and UBC 844 produced 19 total scorable polymorphic and monomorphic bands (Fig. 5). The size of amplified bands ranges between 275 1700 bp. UBC 810 produced maximum of 13 scorable bands, out of which 12 locus exhibited polymorphism and only one exhibited monomorphic band. The PM-MN exhibited 38.46% polymorphism against PM-ML and PM-AP whereas UBC 844 produced 6 clear bands with only one monomorphic band between PM-ML and PM-AP and 5 loci exhibited polymorphism. The PM-MN exhibited no banding pattern in UBC 844

which depicts a high degree of polymorphism with 66.67% against PM-ML and PM-AP.

The PIC value and percentage of polymorphism were also calculated to analyze the molecular marker efficiency. The PIC value for RAPD ranged from 0.00 to 0.44 with an average of 0.12 (Table 4). The highest PIC value was 0.44 observed in primers AP-P-23 and CORR1, PIC value of 0.23 was observed in PER1 whereas the remaining six primers obtained the least PIC value with 0.00. In ISSR, the highest PIC values observed were in UBC-844 with 0.48 and UBC-810 with 0.40. The correlation analysis between RAPD and ISSR markers using normalized Mantel statistics (r) was 0.794 (0.8) which represented a high correlation (Ramakrishnan *et al.*, 2004; Naik *et al.*, 2017; Zhang *et al.*, 2018) and a good degree of fit between the two markers (Haider *et al.*, 2012). According to the marker efficiency test, both RAPD and ISSR were efficient in genetic diversity studies.

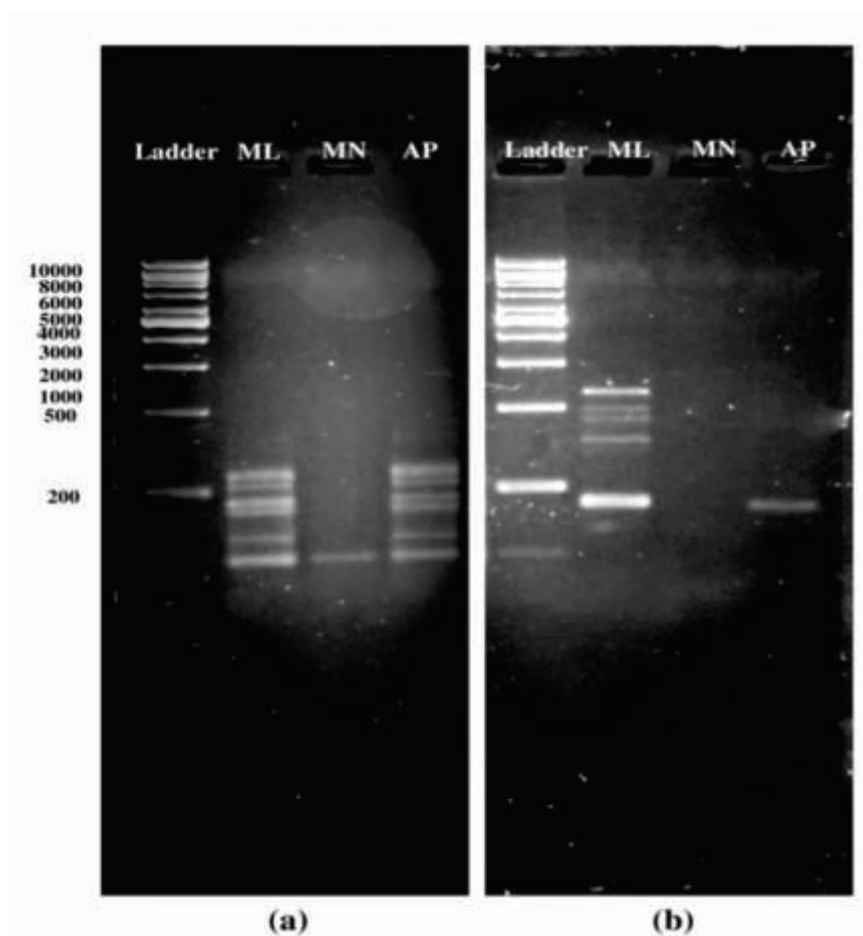


Fig. 5 : PCR- amplicons of three *Pleione maculata* samples by ISSR primers: **(a)** UBC 810 and **(b)** UBC 844, Lane 1- Ladder, lane 2- ML (Meghalaya), lane 3- MN (Manipur), and lane 4- AP (Arunachal Pradesh)

Table 4: Quantitative data of PCR-amplicons by ISSR primers

RAPD	Primer sequence (5 to 3)	Annealing Tm [°C]	No. of scorable bands	No. of poly-morphic bands	No. of mono-morphic bands	% of poly-morphism (p)	Poly-morphic information content (PIC)	Molecular weight (bp)
UBC-810	CT CT CT CT CT CT CT CT RC	52	13	5	1	38.462	0.4012	275-600
UBC-844	GAG AGA GAG AGA GAG AT	52	6	4	1	66.667	0.48	850-1700
Total no of bands			19	9	2		0.44	

Phylogenetic tree

The Nei's genetic distance coefficient matrices within the same population of *P. maculata* ranges between 0.00 to 0.32 distances. The genetic distance between PM-ML and PM-MN was 0.32 distances, PM-ML and PM-AP was 0.04 distances and PM-MN and PM-AP was 0.28 distances as shown in Fig. 6 and Table 5. The phylogenetic tree was based on Nei's genetic distance constructed using UPGMA from NTSYSpc software.

Discussion

A very high genetic variability and isolation within and among populations (Krauss, 1997) is due to distance (Schonswetter *et al.*, 2002) and a new environment (Sreedhar *et al.*, 2007). With the emergence of molecular marker, possibility for gene diversity study and determining genetic relationship within and between species are now successful. The study on gene diversity and structure of populations provides information on the evolutionary status and

Table 5 : Genetic distance between *Pleione maculata* from three different geographical regions based on Nei's distance coefficient obtained from RAPD and ISSR marker analysis.

	PM-ML	PM-MN	PM-AP
PM-ML	0.000		
PM-MN	0.321	0.000	
PM-AP	0.041	0.280	0.000

biological conservation. The commonly known molecular markers use to detect plant gene diversity were RAPD, amplified fragment length polymorphisms (AFLP), simple sequence repeat (SSR) and ISSR etc. RAPD markers have been commonly employed due to their rapid genetic stability assessment in plants, but they sometimes fail to expose repetitive genome sequences in a few plants (Palombi and Damiano 2002). Apart from its application, RAPD marker also has limitations in terms of reliability and reproducibility (Poczai *et al.*, 2013; Poobathy *et al.*, 2013). Therefore, to overcome these limitations an advanced marker has to be employed to verify and clarify the previous marker results. In the present study, the ISSR marker was chosen to validate the RAPD analysis due to its repetitive sequences, stringency, polymorphic, reliability, reproducibility, require a long primer length, high annealing temperature (Raj *et al.*, 2011; Haider *et al.*, 2012), and informative characteristics (Amom *et al.*, 2018; Tikendra *et al.*, 2019).

To resolve the status of the three morphotypes whether they represent ecotypes, subspecies, or distinct

lineages future studies should utilize molecular markers such as ISSR and RAPD. These markers have a strong track record in orchid studies: for instance, *Rhynchostylis retusa* populations in Goa were analyzed using both ISSR and RAPD markers, revealing significant genetic variation (Parab and Krishnan, 2008). In Nepal, *Dendrobium transparens* displayed high genetic stability after micropropagation when analyzed with both marker types (Pant *et al.*, 2022). Similarly, endangered *Dendrobium fimbriatum* var. *oculatum* maintained genetic fidelity in tissue culture, as assessed by RAPD, ISSR, and SCoT markers in a 2021 study (Tikendra *et al.*, 2019) These recent studies underscore the efficacy of these markers for distinguishing genetically stable lineages and verifying clonal fidelity—crucial for conservation and taxonomic clarity.

In the present study, DNA isolation of *P. maculata* was carried out and the estimated length in agarose gel electrophoresis was more against 1kb DNA marker. *P. maculata* from three different geographical regions exhibited differences in phenotypic expression of their pseudobulb which could be due to habitat fragmentation and temperature variation. Due to petiole lines, hard leaf texture, high amount of phenolics, and flavonoids in *P. maculata*, DNA extraction using the conventional method did not provide high quality DNA as compared to column-kit (Sympli *et al.*, 2021).

A molecular analysis of *Coelogyne pandurata* and *Coelogyne rumphii* with their replicates was also studied using RAPD primers OPD-08 producing one monomorphic band each and OPA-13 produced a total

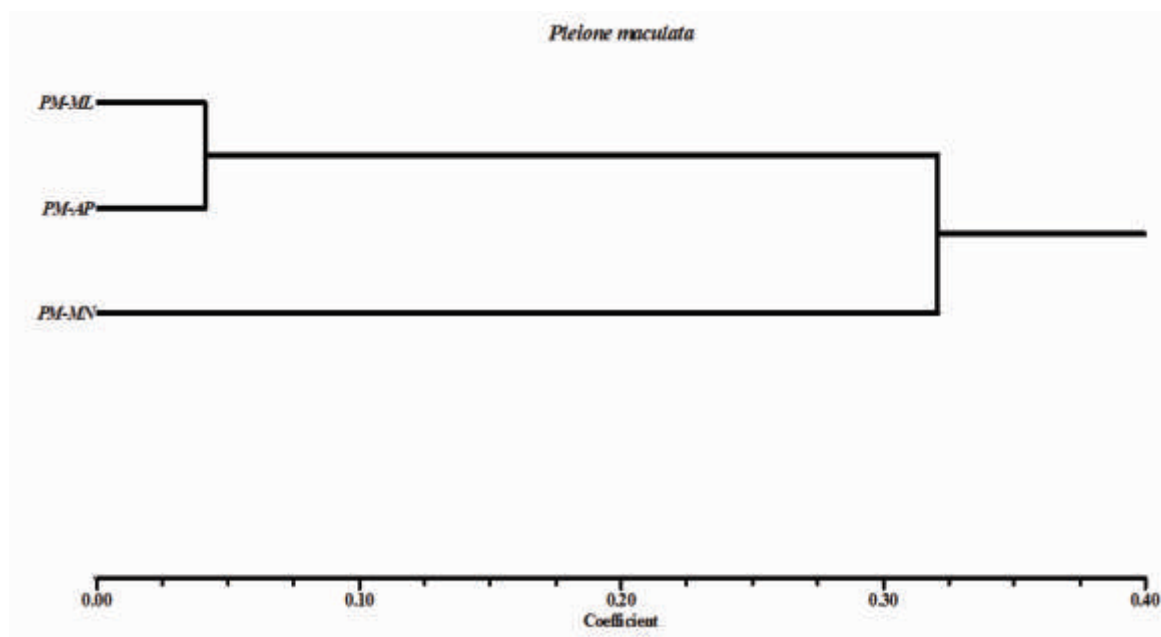


Fig. 6 : UPGMA dendrogram of RAPD and ISSR analysis showing the genetic relationship between *Pleione maculata* from three different geographical regions

of 7 amplified bands with range 400-2000 bp (Hartati and Muliawate 2020). In the present study, RAPD OPD-08 exhibited one monomorphic band each within the three species of *P. maculata* whereas OPA-13 exhibited a total of 15 amplified bands with a range 500-1000 bp. Six *Coelogyne* species, a related genus of *Pleione* were studied using ISSR primer UBC-810 produced 13 total numbers of amplified bands with band size range 250-2000 bp and UBC-844 with 9 amplified bands ranges between 250-1300 bp giving 100% polymorphism (Hartati, 2017). ISSR primers UBC 810 and UBC 844 selected were based on higher polymorphism in epiphytic *Coelogyne* species (Hartati, 2017), a related genus of *Pleione* orchids (Cribb and Butterfield, 1999; Gravendeel *et al.*, 2001; Symplic *et al.*, 2018). The present study also revealed a high level of genetic diversity within species were ISSR marker exhibited 66.67% polymorphism in PM-MN against PM-ML and PM-AP when compared to that of RAPD with 50% polymorphism. UBC-810 exhibited a total of 13 numbers of amplified bands in range 275-600 bp and UBC-844 a total of 6 amplicons in range 850-1700 bp (Table 4). This level of gene diversity might be due to gene flow, random genetic drift, natural selection, gene deletion, habitat fragmentation and isolation (Barrett, 1992; Sreedhar *et al.*, 2007; S. Uebbing *et al.*, 2016). There is a greater capability of species to adapt and evolve when the level of genetic diversity is high (Li and Chen, 2004; Naik *et al.*, 2017) than those species with low level genetic diversity which may eventually fall into rare and extinction category due to decrease in adaptability (Naik *et al.*, 2017). Different molecular markers and different primers of the same marker yielded distinct amplification products, which reflected the polymorphism of the genomic regions (Zhang *et al.*, 2018). A combined RAPD-ISSR banding was performed to increase the validation of results as it screens more segments in the genome (Naik *et al.*, 2017). The genetic distance and similarity between and among species can be predicted as closely related when their coefficient of dissimilarity is small (Lu *et al.*, 2011; Hartati, 2017). In the present study, Nei's genetic distance coefficient between species of *P. maculata* ranges between 0.00 to 0.32 distances which indicated small distances.

The different environment has different and specific climatic characteristics such as temperature and precipitation which lead to progressive minor genetic evolution or differences in the expression of phenotypic traits (Zhang, 2017; Chao *et al.*, 2021). Polymorphic information content (PIC) calculation using Gene-Calculator is a bioinformatics tool for the determination of genetic distance, PIC, and Heterozygosity (H) values based on both dominant and co-dominant markers related especially to biological sciences (Bińkowski and Miks, 2018). PIC detects and measures the polymorphism of a marker locus in linkage studies (Guo and Elston, 1999; Nagy *et al.*, 2012). The Mantel test was performed to check the correlation and to verify the level of congruity

of generated data between the two marker (RAPD and ISSR matrices) techniques (Haider *et al.*, 2012). The result from the above PIC and Mantel test calculated indicates RAPD and ISSR markers are efficient for genetic diversity studies.

The Nei's genetic distance coefficient matrices is a multivariate cluster analysis which provides information on the distances among groups and between sub-groups (Naik *et al.*, 2017). Two major clades or major groups were observed in the tree dendrogram of *P. maculata* as presented in Figure 6. The first major clade was further divided into two minor close sub-clusters PM-ML and PM-AP with a distance of 0.04 (4%) which indicates greater similarity. The second major clade divides PM-MN alone with 0.32 (32%) distances from the root indicating slight genetic variation from PM-ML and PM-AP. The slight differences in clustering distances indicate certain geographical variation (Zhang, 2017).

Conclusion

Molecular markers provide a reliable indication in the selection of best genotypes for breeding line, identification of clones, relation among germplasm, and genetic variation study (Nirmala *et al.*, 2006). Therefore, the present study provides a brief foundation on which geographical region of *P. maculata* are closely similar and can be of great importance for selection and plant cross-breeding to conserve them. Due to rare and endangered nature of medicinally important *P. maculata*, awareness toward the conservation and breeding strategies must be implemented to understand its population structure and diversity.

RAPD and ISSR markers proved to be an efficient DNA fingerprinting and molecular characterization tools in determining the relationships, classifications, differences, and similarities within genus of the same species. The generated information on gene diversity in *P. maculata*, a germplasm collection can be established. RAPD and ISSR markers used generated sufficient variability to differentiate and check the similarity within the species. The *P. maculata* species from different geographical regions exhibited a split and good degree of divergent within them. In structural genomics, variation within and among species might be due to gene deletions, duplication or inversions causing phenotypic differences. Preservation of endangered species can be overcome by studying the genetic diversity, which is extremely valuable, as loss of genetic variability may reduce survival chances in the wild. The study found that genetic diversity within the three different geographical regions of *P. maculata* ranges between 0.041 to 0.32 distances which identify that habitat fragmentation does affect the level of gene diversity between populations of *P. maculata*. This study also concluded that a cross between PM-ML and PM-AP might have the highest probability of success as they have a small genetic distance. The study will provide a base idea for

researchers with future objectives to understand the genetic diversity of *P. maculata* and create awareness of its conservation.

रैंडम एम्प्लीफाइड पॉलीमॉर्फिक डीएनए (RAPD) और इंटर सिंपल सीक्वेंस रिपीट (ISSR) मार्कर का उपयोग करके विभिन्न भौगोलिक आवासों से संकटग्रस्त औषधीय एपिफाइट प्लीओन मैक्युलाटा की आनुवंशिक विविधता का विश्लेषण
हकानी डी. सिम्पली और वेदांत विक्रम बोरा

सारांश

प्लीओन मैक्युलाटा एक संकटग्रस्त औषधीय एपिफाइट है, क्योंकि इसके स्प्रूडोबल्ब का उपयोग सिरदर्द, पेट दर्द और यकृत रोगों के इलाज के लिए किया जाता है। संरक्षण और प्रजनन के लिए प्रासंगिक आणविक मार्कर प्रणाली का उपयोग करते हुए प्लीओन मैक्युलाटा के आनुवंशिक विविधता अध्ययनों पर वर्तमान में कोई रिपोर्ट उपलब्ध नहीं है। इसलिए, इस अध्ययन में रैंडम एम्प्लीफाइड पॉलीमॉर्फिक डीएनए (RAPD) और इंटर सिंपल सीक्वेंस रिपीट (ISSR) मार्करों का उपयोग करके तीन अलग-अलग भौगोलिक वितरणों से प्लीओन मैक्युलाटा में आनुवंशिक परिवर्तनशीलता और अंतर-विशिष्ट समानताओं के पैटर्न का आकलन किया गया। मूल्यांकन किए गए 16 RAPD और 2 ISSR मार्करों से, कुल 96 विशिष्ट और पुनरुत्पादनीय डीएनए एम्प्लीकॉन उत्पन्न हुए। रूपात्मक असमानता मैट्रिक्स की गणना मेंटल परीक्षण और नी के आनुवंशिक विविधता गुणांक का उपयोग करके की गई। RAPD के लिए उच्चतम परिकलित PIC मान 0.44 था, जबकि ISSR के लिए उच्चतम PIC मान 0.48 थे। उपयोग किए गए आणविक मार्करों के दो सेट अत्यधिक सहसंबद्ध थे, जिनका सहसंबंध मेंटल सांख्यिकी (r) 0.79 था, जो जनसंख्या आनुवंशिक अध्ययनों में उच्च मार्कर दक्षता दर्शाता है। इस अध्ययन के परिणाम आनुवंशिक संबंध, आनुवंशिक विविधता और पी. मैक्युलाटा जनसंख्या के भीतर प्रजातियों के संरक्षण, प्रबंधन, प्रजनन और संकर उत्पादन के लिए जर्मप्लाज्म संग्रह स्थापित करने की रणनीतियों को समझने का आधार प्रदान करेंगे।

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