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RESEARCH ARTICLE

Genetic Variation and Gene Flow Estimation of *Nepenthes khasiana* Hook. F- A Threatened Insectivorous Plant of India as Revealed by RAPD Markers

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Abstract

Random Amplified Polymorphic DNA (RAPD) markers were utilized for determination of diversity within and among the three populations of *Nepenthes khasiana* Hook f., a threatened insectivorous plant of Meghalaya (India). A total of 90 bands were generated from 10 random amplification polymorphic DNA (RAPD) primers of which 71 were found to be polymorphic (78.89%). Nei's gene diversity (*h*) ranged between 0.124 - 0.201 with overall diversity of 0.228 while Shannon's information index (*I*) values recorded between 0.187 - 0.308 with an average value of 0.352. The values of gene flow (*Nm* = 1.284) and the diversity among populations (0.280) recorded demonstrates higher genetic variation within the population. AMOVA analysis revealed a low level of genetic variation (21.96%) among the populations. This study indicates that some variation still exists within and between the existing populations of *N. khasiana*, thus, these populations could provide materials for re-establishing of this important rare and threatened species.

Key words: conservation, genetic diversity, gene flow, *Nepenthes khasiana*, RAPD

Introduction

Nepenthes khasiana Hook f. (Common name: English: Pitcher plant) was discovered by Sir J. D. Hooker in the year 1873. It belongs to the family Nepenthaceae with polyploid chromosome number 2n = 80. The genus *Nepenthes* comprises 86 species distributed in different areas of the world (www.joachim_nerz.de/nepintro, Jebb and Cheek 1977). *N. khasiana* is the only species found in India. It is restricted to a few areas of Meghalaya, situated in northeast India. *N. khasiana* is a plant which is of botanical curiosity as well as ecological importance. *N. khasiana* is a climbing undershrub which ranges from a few centimeters to several meters in height (Figs. 1A and B). The presence of the pitchers renders ornamental importance to this plant; therefore it is extensively collected for trade. The plant has some ethno-medicinal uses. The local physicians, known as the 'Kavirajas', prescribe the pitcher fluid for diabetic patients. Pitcher as well as pitcher powder is used to treat patients with cholera, frequent and painful urination, cysts, vaginal tumors, and leprosy (Bordoloi 1977). The population of *N. khasiana* has dwindled in the last few decades due to deforestation and forest fires, excessive collection for trade, and slash and burn agricultural practice locally known as 'jhum' cultivation. It is now threatened in its natural habitat. *N. khasiana* is listed as an endangered plant in Appendix I of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) (Bordoloi 1977). In view of the importance of conserving this endemic and endangered pitcher plant, knowledge of genetic diversity within the genus is essential for establishing effective and efficient conservation practices. Although there were some attempts to record natural genetic variation in *N. khasiana* using molecular markers (Bhau et al. 2009), the precise estimation of genetic diversity and gene flow in populations which pro-

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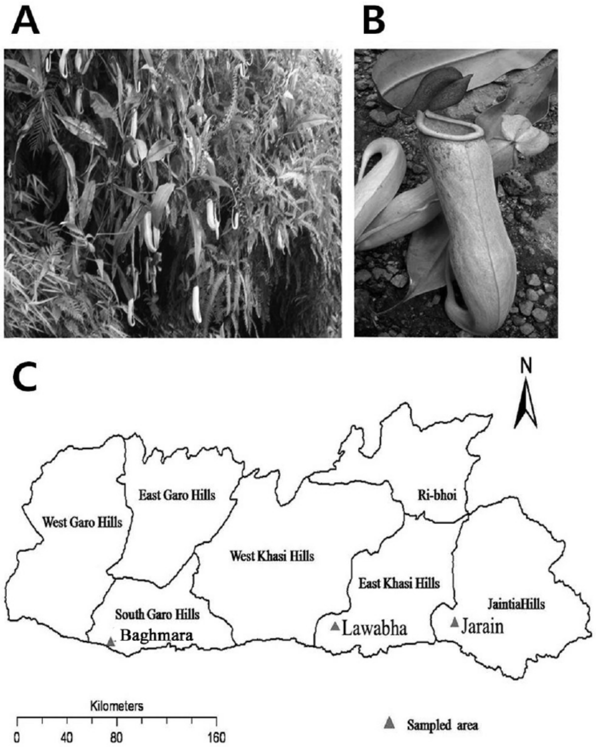

Fig. 1. *Nepenthes khasiana* plants growing in natural habitat (A); closer view (B); details of populations sampled from Meghalaya (C).

vides an insight on the patterns of adaptation of a species in fragile ecosystems is lacking. Therefore, the main objective of the present investigation was to evaluate the genetic diversity and gene flow both within and among the three populations of *N. khasiana* collected from three different habitats of Meghalaya (India), using RAPD (Random Amplified Polymorphic DNA) markers.

Materials and Methods

Three natural populations located in different parts of Meghalaya viz. Jarain (Jaintia Hills District), Lawabah (East Khasi Hills District), and Baghmara (South Garo Hills District) were selected (Fig. 1C). Nine individuals per population (27 individuals) were randomly selected for the present study

DNA extraction and RAPD analysis

Leaf samples from these plants were collected and immediately frozen in liquid nitrogen until DNA extraction was performed. Total genomic DNA was extracted from young leaves following the modified CTAB method (Porebski et al. 1997). Genomic DNA was quantified against a known quantity of unrestricted Lambda (λ) DNA by electrophoresis using 0.8% agarose gel.

Four kits (A, C, K, and H) comprising 80 decamer random primers per kit (Operon Technologies, Alameda, CA, USA) were screened out for the present investigation. To optimize the PCR amplification conditions, experiments were carried out with varying concentrations of DNA template, and primers, *Taq* polymerase, $MgCl_2$ as well as dNTPs. A total of 10 primers were used for PCR-RAPD analysis. All the PCR reactions were carried out in 25 µL volumes containing 20 ng of template DNA, 0.2 mM of each of the four dNTPs, 1X PCR buffer (10 mM Tris pH 9.0, 50 mM KCl), 1.5 mM $MgCl_2$, 0.5 U *Taq* polymerase (Bangalore Genei, India) and 10 pmol of primer. The reaction programmes were set at 94ºC for 5 min followed by 40 cycles of 94ºC for 1 min, 34ºC for 1 min, 2 min elongation at 72ºC, and a final extension at 72ºC for 15 min in a thermal cycler 2720 (Applied Biosystems, USA). After completion of the amplification, 2.5 µL 10X blue dye was added to the samples, and the amplified DNA was analyzed on 1% agarose gel in 1X TAE buffer at 65 - 70 V for 4 - 5 h. The gels were observed on the gel documentation system (UVP Gel Doc) and photographed.

Data analysis

All amplification products produced through RAPD were scored as '1' for presence and '0' for absence across 27 accessions representing three populations. Molecular weights of the bands were estimated by using *Hind*III digested λ-DNA ladder as standards. The binary data matrix, thus obtained was used for further analyses of polymorphic loci. Genetic diversity parameters including the percentage of polymorphic loci (Pp), Nei's gene diversity (*h*), and Shannon index (*I*) were calculated with POPGENE version 1.31 (Yeh et al. 1999) to estimate genetic variation level. Gene flow (*Nm*) was estimated from the $Nm = 0.25 \times (1- G_{st}) / G_{st}$. Analysis of Molecular Variance (AMOVA) was performed using Arlequin version 3.01 (Excoffier et al. 2005) at three hierarchical levels to examine differences among and within populations. The significance of this analog was evaluated by 1,000 random permutations of sequences among populations (Miller 1998). Data were analyzed using simqual route to generate Jaccard's similarity coefficient with NTSYS pc Versions 2.02k programme (Rohlf 1998). Dendogram was prepared for all the individuals using UPGMA and SAHN clustering for analysis of genetic relationships for all the collections. The ability of the primers to distinguish between accessions was assessed by calculating their Resolving power (*Rp*) (Prevost and Wilkinson 1999) for RAPD primers. This function has been found to correlate strongly with the ability to distinguish between genotypes and the formula: $Rp = \Sigma Ib$, where band informativeness, $Ib = 1 - (2 \times |0.5 - p|)$ and p is the proportion of accessions containing band I.

Results

A total of 90 bands were generated from 10 RAPD primers, of which 71 were found to be polymorphic (78.89%) (Table 1). The number of fragments amplified by each primer ranged from 7 (OPK-04 and OPH-13) to 11 (OPK-19). The total *Rp* value of the primers was calculated to be 110.592, ranging from 7.333 (OPC-15) to 16.074 (OPH-03) (Table 1). RAPD marker profiles

Table 1. Data of RAPD primers used in the present study and the extent of polymorphism

Sl No.	Primer Name	Total No. of bands	No. of polymorphic bands	No. of monomorphic bands	% of Polymorphisim (P)	Resolving Power (*Rp*)
1	OPC-15	9	7	2	77.78	7.333
2	OPH-03	10	9	1	90.00	16.074
3	OPH-13	7	3	4	42.86	12.667
4	OPH-20	8	5	3	62.50	10.740
5	OPK-04	7	6	1	85.71	7.926
6	OPK-07	10	8	2	80.00	14.148
7	OPK-16	10	7	3	70.00	12.889
8	OPK-17	10	8	2	80.00	10.963
9	OPK-18	8	7	1	87.50	9.556
10	OPK-19	11	11	0	100	8.296
	Total	90	71	19	78.89	110.592

produced by the primer OPK-19 is shown (Fig. 2A). The observed number of alleles (*Na*) and effective number of alleles (*Ne*) ranged between 1.367 - 1.667 and 1.208 - 1.338, respectively. Similarly, Nei's gene diversity (*h*) and Shannon's Information index (*I*) ranged between 0.166 - 0.201 with overall diversity of 0.228 and 0.187 - 0.308 with an average value of 0.352, respectively (Table 2). Percentage of polymorphic loci (Pp) was estimated in the range of 33.67 - 66.67% with relatively high polymorphic loci (78.89%) among the 27 individuals. The gene flow value and the diversity among population were found to be 1.284 and 0.280, respectively (Table 2). Analysis of AMOVA ($P < 0.001$) showed that most of the genetic variation (78.04%) was observed within the populations, whereas the variance among populations was 21.96 %.

The similarity coefficients ranged from 0.46 to 0.90 and the dendogram based on similarity index of 27 individuals indicated that the sample from the same populations did not form a distinct group (Fig. 2B)

Discussion

Maintenance of both genetic diversity and population distinctiveness is a major goal in the conservation management plans of overexploited species (Meffe 1994). The information about population genetic structure is a key to rational use and conservation of resources because it contributes to understanding the processes by which a species organizes genetic variation in local populations, and the patterns of variation among populations and species. The present investigation using RAPD marker revealed that in *N. khasiana* most of the genetic variation (78.04%) was observed within the populations, whereas the variance among populations was 21.96%. Hogbin and Peakall (1999) noted that variance among populations varied from 0 to 43%, the higher values being recorded in species known to undergo selfing. The relatively low diversity partitioned among populations in *N. khasiana* is, therefore, not surprising as it is a dioceous hence cross-pollinated species. It has been reported that outcrossing species tend to have higher levels of variability within populations but smaller degrees of differentiation among populations than selfing species (Hamrick and Godt 1989; Schoen and Brown 1991). Even so, a high range of genetic diversity was reported between different populations of *N. mirabilis* Druce from different geographical areas of northeastern, central, and southern Thailand by Chaveerach et al. (2006) using ISSR markers. Similarly, the great diversity of *N. khasiana* at intra-specific level may be because of isolation of different populations within Meghalaya (Bhau et al. 2009).

A relatively high value of percentage of polymorphic loci (78.89) among the 27 individuals and with a moderate polymor-

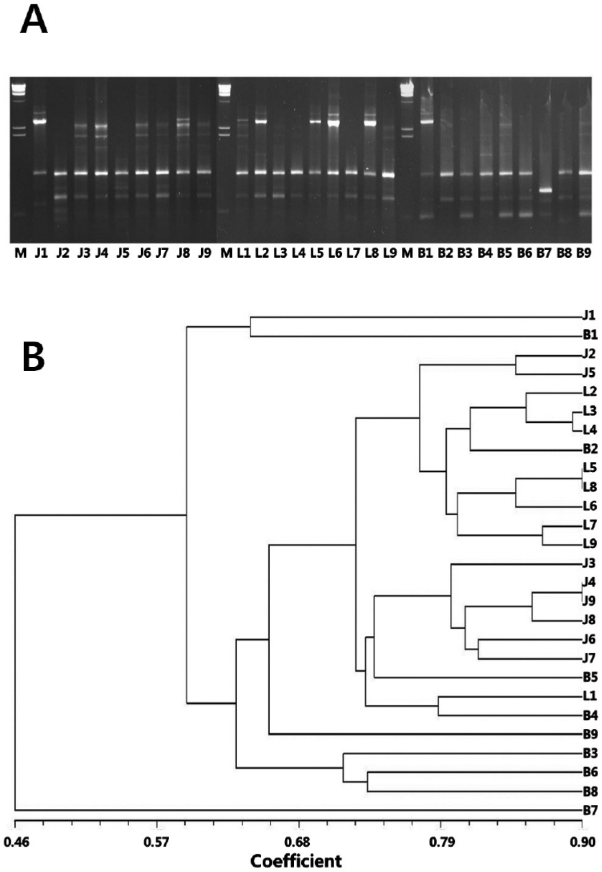

Fig. 2. (A) RAPD marker profiles of *Nepenthes khasiana* populations generated with RAPD OPK-19 primer, M-marker- *Hind* III digested l-DNA; J11-J9 represents population from Jarain, L1-L9 represents population from Lawabha and B1-B9 represents population from Baghmara; (B) UPGMA clustering of *Nepenthes khasiana* populations.

Table 2. Genetic diversity static and differentiation parameters for three wild populations of *N. kahasina*; *h* = Nei's (1973) gene diversity; *I* = Shannon's Information index (Lewontin 1972); *Na* = Observed number of alleles; *Ne* = Effective number of alleles (Kimura and Crow 1964); *Pp* = percentage of polymorphic loci (Lynch and Milligan 1994); SD = Standard deviation; G*st* = diversity among populations, *Nm* = gene flow 0.25 (1 – G*st*)/G*st*; H*pop* = variability within population, H*sp* = Total variability; *Ss* = No. of individuals

Population	*Ss*	*Na* ± SD	*Ne* ± SD	*h* ± SD	*I* ± SD	*Pp* (%)	*Np*	*Hsp*	*Hpop*	*Gst*	*Nm*
Jarain (Jaintia Hills)	9	1.533 ± 0.501	1.282 ± 0.367	0.166 ± 0.195	0.252 ± 0.276	48.00	53.33				
Lawabha (East Khasi Hills)	9	1.367 ± 0.485	1.208 ± 0.327	0.124 ± 0.183	0.187 ± 0.2659	33.67	36.67				
Baghmara (South Garo Hills)	9	1.667 ± 0.474	1.338 ± 0.366	0.201 ± 0.191	0.308 ± 0.269	66.67	60.00				
Overall population	27	1.788 ± 0.410	1.368 ± 0.330	0.228 ± 0.178	0.352 ± 0.248	78.89	71.00	0.228	0.164	0.280	1.284

phism in all the populations; also the overall high gene flow (*Nm*) rate (1.284) as reported in the present study confirms the existence of genetic variation within population levels. Genetic differentiation depends on the scale of environmental heterogeneity and the balance of selection and higher gene flow (Kumar et al. 2011; Savolainen et al. 2007; Sharma et al. 2011). The study of sequence variation also provides means to detect loci responsible for local adaptation (Wright and Gaut 2005). Individual genes or polymorphisms are the heritable units of adaptation because recombination decouples linked genes and different genes occur on different linkage groups. When populations become locally adapted to contrasting environments, alleles entering a new population may be eliminated by selection, such that the gene flow is partly restricted. The stronger the selection, the more rapidly immigrant alleles of lower fitness will be eliminated from the population thereby reducing effective migration rates and increasing the time to coalescence (Charlesworth et al. 1997). However, gene flow will continue to occur at unlinked loci until populations become reproductively isolated, and much of the genome may be undifferentiated if dispersal rates and gene flow levels are high, besides intra- and inter-specific competition limit the distribution of genotypes.

The present results indicate that RAPD can be used to establish the relationships among the different populations and even individuals within the existing populations. RAPD data from this study indicate that some variation still exists within and between the existing populations of *N. khasiana*, thus, these populations could provide materials for re-establishing other populations of this important rare and threatened species. Furthermore, the high genetic diversity within populations indicate that the most effective strategy for preserving genetic variation would be to protect the existing populations *in situ* in order to preserve as much genetic variation as possible, especially for those populations with higher genetic diversity.

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