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RESEARCH ARTICLE

PCR based molecular characterization of *Nepenthes khasiana* Hook. f.—pitcher plant

B. S. Bhau · K. Medhi · T. Sarkar · S. P. Saikia

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Abstract *Nepenthes khasiana* Hook. f. belonging to monotypic family Nepenthaceae is a rare, endangered, dioecious member of the carnivorous plant found in North-East India. The plant is endemic to the Indian state of Meghalaya and is distributed throughout the state from West Khasi hills to East Khasi hills, Jaintia hills and East, West to South Garo hills from 1,000 to ca. 1,500 m altitude. Multi-locus analysis using PCR based Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers were used for the first time to assess the genetic diversities of *N. khasiana* Hook. f. collected from different parts of Meghalaya. It was observed that RAPD analysis showed more polymorphism than ISSR fingerprinting in revealing genetic polymorphism in *N. khasiana* Hook. f. The result of cluster analysis by using UPGMA method showed that the groups based on pooled RAPD–ISSR genetic similarity were more similar than the groups based on RAPD. Furthermore, genetic similarity reveals variability within the population at Jarain of Jaintia hills, while between populations the Baghmara region differs from the others with at least 40% dissimilarity. The results show a broad range of genetic diversity within the populations of *N. khasiana* Hook. f.

Keywords Conservation · Genetic diversity · ISSR · Molecular markers · *Nepenthes khasiana* Hook. f. · Pitcher plant · Polymorphism · RAPD

Introduction

Carnivorous plants are interesting botanical oddities that show a range of characters different from those of a normal plant. These plants typically inhabit environments where nutrients are limited, and insects trapping may have evolved as a means of providing a supplemental source of nutrients, especially nitrogen (Givnish et al. 1984; Gallie and Chang 1997). Out of different kinds of carnivorous plants spread around the world, single species of *Nepenthes* i.e., *Nepenthes khasiana* Hook. f. are found in India (Kanjilal et al. 1940).

Nepenthaceae is represented by a single genus *Nepenthes* which is popularly known as tropical pitcher plant (Mokkamul et al. 2007). Indo-Malaysia is considered as the center of evolution of the genus *Nepenthes*. Many species of this genus are either endemic to certain areas or have a very restricted distribution.

B. S. Bhau and K. Medhi contributed equally.

B. S. Bhau (✉) · K. Medhi · T. Sarkar · S. P. Saikia
Plant Genomics laboratory, Medicinal Aromatic and Economic Plants (MAEP) Division, North-East Institute of Science and Technology (NEIST)-Jorhat (Formerly RRL), Jorhat, Assam 785006, India
e-mail: bsbhau@gmail.com; bhaubs@rrljorhat.res.in

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Some biodiversity hotspots are significant in having their endemic species concentrated in exceptionally small areas (Myers et al. 2000). Meghalaya, in India is one of such hotspots for *Nepenthes khasiana* Hook. f. The species and its habitats are unique, as it occurs as isolated populations within Meghalaya. It is a rare, endemic, endangered, dioecious insectivorous climbing pitcher plant (Fig. 1a) of great biological and ecological importance (Jain and Sastri 1980; Hooker 1886; Mao and Kharbuli 2002). It is found growing from West Khasi hills to East Khasi hills, Jaintia hills, and east to west and south Garo hills from 1,000 to ca. 1,500 m altitude. It occurs in the Jarain area of Jaintia Hills and the Baghmara, Bandari, Chokpot area of the Garo hills, and few more localities, such as Jarain, Bhagmara, Nongstoin, Mukthapur, Bhagmara, Lawbahand and Sonapahar (Joseph and Joseph 1986). It can be said that the species represents ancient endemic remnants of older flora which usually occur in land masses of geological antiquity (Paleoendemics), (Bramwell and Valentine 1972).

The species is under major threats due to many human activities, such as road construction, agriculture, deforestation; coal mining, animal grazing, landslides etc., destroying natural habitats of this rare endemic plant. The plant is also being collected and exported by local plant collectors to other states of India. On account of its fascinating beauty of pitcher (Fig. 1b), *N. khasiana* Hook. f. is often purchased from the markets and hybridized to produce a diversity of pitcher characters (Mao and Kharbuli 2002). Hence the species is of great botanical and horticultural interest (Khoshbakht and Hammer 2007; Mukerjee et al. 1984).

The fluid of the unopened pitcher of *N. khasiana* Hook. f. is used by local Khasi's and Garos as an eye drop for redness, itching, to cure cataract and night blindness and is also taken for stomach troubles, diabetes and for female diseases (Kumar et al. 1980; Rao et al. 1969; Joseph and Joseph 1986). The unopened pitcher with its content is made into a paste and applied for various skin diseases, including leprosy (Rao et al. 1969), sometimes mixed with rice beer and is taken to ease urinary troubles and blockages (Kharkongar and Joseph 1981). Also the pitcher along with the insects within it is made into paste mixed with water and given to cholera patients. Hence the species is an important ethno medicinal plant and needs to be conserved (Mao and Kharbuli 2002). The genus has also been studied for their staining properties of chemical nepenthoquinone. Plumbagin, one kind of nepenthoquinone has been reported from *Nepenthes* of Nepenthaceae (Cannon et al. 1980). Other possible roles of Nepenthoquinone are as allelopathic substance (Harbone 1982; Reynolds 1987; Jayaram and Prasad 2005), or as insecticidal, molluscicidal or antifeedant chemicals (Thomson 1987). The species is officially classified as a threatened species and is included in the list of

Fig. 1 **a** *Nepenthes khasiana* plant growing in the natural habitat; **b** Close-up of a pitcher

Fig. 2 Collection sites within Meghalaya

rare and threatened taxa of India (Jain and Baishya 1977; Jain and Sastri 1980). It is also included in the Appendix I of the Convention of International Trade of Endangered Species (CITES) of the world, which means that it is a protected species and any type of collection from the wild and its sale is forbidden except when the purpose of the import is not commercial, e.g., for international scientific exchange.

In view of the importance to conserve this beautiful, endemic and endangered pitcher plant, knowledge of genetic diversity within the genus is essential for establishing effective and efficient conservation practices. Traditionally, morphological characters have been used to characterize levels and patterns of diversity. Since these traits represent only a small portion of the plant genome and are influenced by environmental factors, plant age, phenology, they have limited utility for describing the potentially complex genetic structure, which may exist within and in between taxa (Avise 1994). These facts make difficult the use of such descriptions in plant identification and screening the genetic relationships. Various molecular approaches have been devised to overcome these constraints (Soltis and Soltis 1991). The present study was aimed at determining the extent of genetic diversity within and among populations.

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). A number of generalizations have been made about relationships among the level and distribution of genetic variation of a species and its ecological and biological traits (Loveless and Hamrick 1984; Lewontin and Hubby 1966; Heubl et al. 2006). However, such generalizations have not always been supported by the available information. In rare plant species (Young et al. 1996; Gitzendanner and Soltis 2000; Hoebee and Young 2001). Hence, there is a need to develop empirical information about the extent and distribution of genetic diversity in species that are targeted for conservation. In this paper, we provide baseline data on RAPD and ISSR variations within and among the seven populations of *N. khasiana* Hook. f. and attempt to identify factors that may have influenced the partitioning of genetic diversity. On the basis of this information, we then can recommend a conservation programme for this species.

Materials and methods

Study sites and sample collection

Six natural populations located in different parts of Meghalaya were selected for the evaluation of RAPD analysis. Young leaves of 45 individuals of *N. khasiana* Hook. f. were collected from populations in Bhagmara (BM), Jarain (JR), Rongsai (RSN), Baghmara Massighat (BMG), Chokpot (CP) and Bandari (BD). The localities are shown on the map (Fig. 2). Another site of collection was at the North-East Institute of Science and Technology (NEIST), Jorhat campus. Leaf material were immediately dried using self-indicating silica gel, then transported to the lab, and stored at −45°C until DNA extraction.

DNA isolation and PCR amplification

Leaves were freeze-dried using a lyophilizer. DNA extraction of the samples was done by modified

CTAB method (Doyle and Doyle 1990). Genomic DNA was quantified against a known quantity of unrestricted Lambda (λ) DNA by running it in a 0.8% agarose gel.

RAPD amplification

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 12 primers were used for PCR–RAPD analysis. The primers were from the OPA, OPAA, OPO, OPBH, OPP, OPC, and OPB, series of Operon technologies (Alamenda, CA, USA) with more than 60% G + C as reported by Lim et al. (2000) for better RAPD profile in *Nepenthes*.

Amplification was performed on a PXE 0.02 Thermalcycler (Thermo electron corporation) with 15 µl reaction mixtures containing 0.05 µg of template DNA, 0.4 mM of each dNTPs (dATP, dTTP, dCTP and dGTP), 0.25 µM of Taq DNA Polymerase, 10 pmol of each primer and 2.4 mM of $MgCl_2$ (all from Fermentas, MBI). The amplification regime was performed with the following programmes: −95°C for 3 min followed by 35 cycles with 95°C for 1 min, 35°C for 2 min, 72°C for 2 min and a final extension at 72°C for 10 min.

Amplified PCR products were performed onto an agarose (1.5% w/v) gel electrophoresis in 1× TBE buffer at 70 V for 150 min. The gel was visualized by Ethidium Bromide staining and photographed under UV light by a gel Doc System (G Box HR, Syngene, UK).

ISSR amplification

PCR reactions were conducted in volumes of 15 µl containing 0.05 µg of template DNA, 0.4 mM of each dNTPs (dATP, dTTP, dCTP and dGTP), 0.25 µM of Taq DNA Polymerase, 10 pmol of each primer and 2.4 mM of $MgCl_2$ (all from Fermentas, MBI). PXE 0.02 Thermalcycler (Thermo electron corporation) was used with the programmed set at 95°C for 3 min followed by 35 cycles with 95°C for 1 min, 35°C for 2 min, 72°C for 2 min and a final extension at 72°C for 10 min. Amplified PCR products were performed onto a 1.8% (w/v) agarose gel electrophoresis in 1× TBE buffer at 70 V for 200 min.

Data analysis

Each gel of RAPD and ISSR was analyzed by scoring present (1) or absent (0) bands. The RAPD, ISSR and pooled data matrices were entered into the NTSYS version 2.02 K package. Pair wise comparisons of populations were made and genetic diversity parameters were determined. Genetic similarities based on Jaccard's coefficient were calculated among all possible pairs using the SIMQUAL option and ordered in a similarity matrix for all the three types of data matrices. A dendrogram was constructed by employing UPGMA (Unweighted Pair Group Method with Arithmetic Average) (Sokal and Sneath 1963) to group individual into discrete clusters. The ability of the primers to distinguish between accessions was assessed by calculating their Resolving power (Rp) (Prevost and Wilkinson 1999) for both RAPD and ISSR primers. This function has been found to correlate strongly with the ability to distinguish between genotypes and the formula: $Rp = \sum I_{b,}$ where band informativeness, $I_b = 1-(2 \times |0.5-p|)$ and p is the proportion of accessions containing band I. Mantel matrix-corresponding test (Mantel 1967) was carried out to compare the RAPD and ISSR data matrices.

Results

RAPD markers

About hundreds primers (Operon Technologies, US) were screened for satisfactory application and twelve primers were finally selected. A total number of 149 fragments ranging in size from 2,800 to 150 bp were amplified of which 139 (88.59%) were polymorphic (Fig. 3a). The number of fragments amplified by each primer ranged from 9 (OPP-18) to 20 (OPBH-08 and OPP-08) (Table 1). There is only 11.41% of sharing bands. The Jaccard's coefficients of similarity varied from 0.978 to 0.376. The dendrogram derived from UPGMA cluster analysis indicates 4 groups of the 48 genotypes, branching at a similarity value of 61% (Fig. 4a). Group1 includes 40 genotypes from all the seven sites of collection whereas group 2 comprises 5 genotypes from Jarain only. Group 3 contains a single genotype from NEIST, Jorhat and the 4th group includes 2 genotypes, from Baghmara and Baghmara

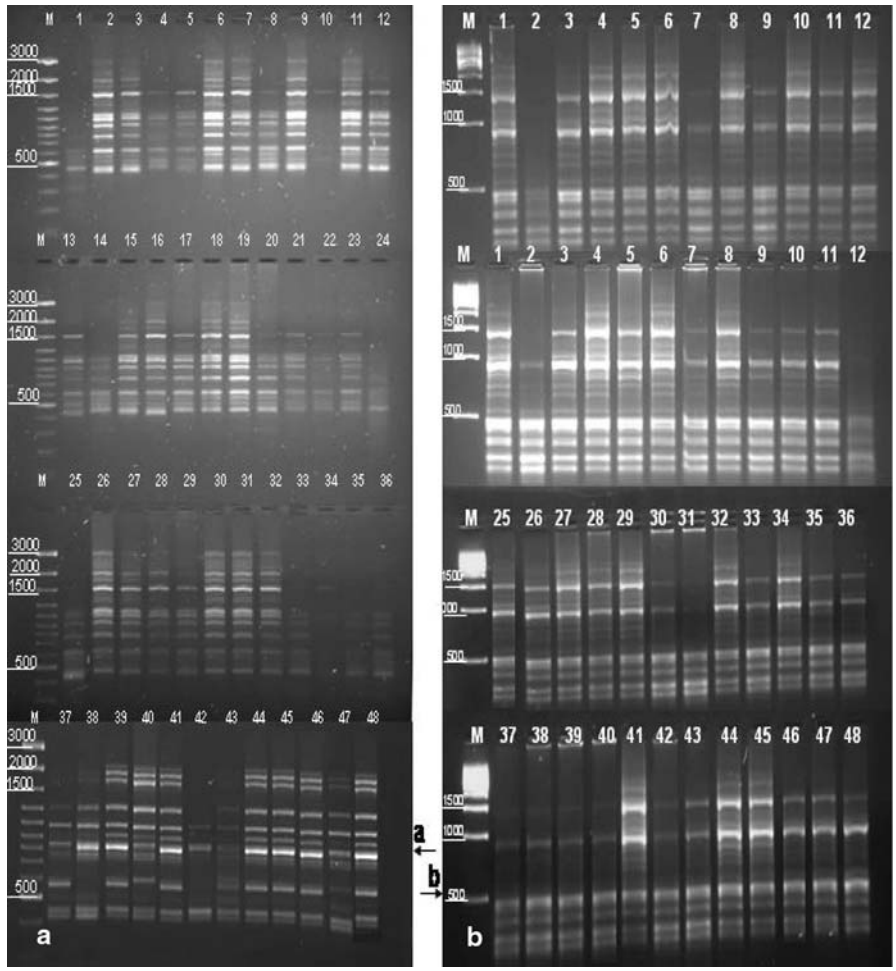

Fig. 3 Banding profile in *N. khasiana.* **a** RAPD Primer OPP-12; **b** and ISSR primer 811

Massighat. The Rp values of the primers used represents a total of 68.28, ranging from 2.8 (OPBH-08) to 9.7 (OPP-15). Characteristics of various RAPD markers like MB (monomorphic bands), PB (polymorphic bands), PPB (Percent polymorphic bands), Rp values etc., were given in Table 1.

ISSR markers

About Eleven ISSR primers from Clonitec were used for producing 107 fragments of which 74 were polymorphic (69.16%). The amplified fragments ranged from 5,000 to 100 bp with an average of 9.7 fragments per primer (Fig. 2b; Table 2). Jaccard's similarity coefficient varied from 0.50 to 0.96. UPGMA cluster analysis reveals 3 groups at a similarity coefficient of 65% (Fig. 4b). Group 1 consists of 22 genotypes from Jarain, Rongsai, Bandari and Baghmara only while group 2 consists of 18 genotypes from all the seven collection sites including all genotypes of Chokpot in this group and the 3rd group consists of 8 genotypes from Jarain, Bandari, Rongsai, Baghmara Massighat and NEIST. The collective Rp value of the primers was 41.58, ranging from 1 (826) to 6.98 (808 and 811). Characteristics of various RAPD markers such as MB (monomorphic bands), PB (polymorphic bands), PPB (percent polymorphic bands), Rp values etc., were given in Table 2.

Combined study of RAPD and ISSR

Both RAPD and ISSR data matrices were combined to calculate out the Jaccard's coefficient. The similarity coefficient ranged from 0.96 to 0.50. The cluster analysis was carried out by using UPGMA method.

Table 1 Description of various characteristics of RAPD marker

Primer	MB	PB	Total	Length of amplicons (bp)	PPB	Rp value
OPBH-08	1	8	9	1,500–400	88	2.8
OPP-19	1	12	13	600–150	92	5.5
0PC-11	4	6	10	800–150	60	4.4
OPBH-03	2	9	11	1,500–350	81	4.6
OPP-07	1	12	13	2,500–350	92	5.9
OPP-15	2	18	20	1,800–200	90	9.7
0PP-12	3	15	18	3,000–400	83	7.5
OPP-08	1	8	9	1,800–350	88	4.7
OPP-4	0	12	12	2,500–400	100	5.1
0PP-09	0	13	13	2,500–500	100	8.5
OPBH-16	1	9	10	1,500–400	90	4.1
OPBH-20	1	10	11	1,800–350	90	5.5
TOTAL	17	132	149			68.28

MB monomorphic bands, *PB* polymorphic bands, *PPB* percent polymorphic bands

Table 2 Description of various characteristics of ISSR marker

Primer	MB	PB	Total	Length of amplicons (bp)	PPB	Rp value
807	0	4	4	550–100	100	1.58
808	3	13	16	1,700–100	81.25	6.98
809	5	4	9	1,300–200	44.44	3.1
811	4	11	15	2,500–100	73.33	6.98
812	0	9	9	1,300–300	100	3.24
825	10	3	13	5,000–500	23.07	2.14
826	2	3	5	1,000–250	60	1.0
827	6	4	10	4,000–500	40	1.84
828	3	5	8	2,000–400	62.5	3.42
834	0	9	9	3,000–1,500	100	5.38
835	0	9	9	3,000–1,700	100	5.92
TOTAL	33	74	107			41.58

MB monomorphic bands, *PB* polymorphic bands, *PPB* percent polymorphic bands

The dendrogram reveals 4 groups where group 1 consist 30 genotypes from Jarain, Rongsai, Bandari, Baghmara Massighat and Chokpot. Group 2 includes 15 genotypes from Chokpot, Baghmara Massighat, Baghmara, Jarain and NEIST, Jorhat and groups 3 and 4 were the same as in the clustering of RAPD (Fig. 4c).

The correlation coefficient between data matrices of RAPD and ISSR based on Mantel test was significant ($r = 0.47006$) but the value of determination coefficient was relatively low i.e., $r^2 = 0.2209$.

Discussion

Because of its dioecious breeding system, genotypic diversity of seedlings founding a new population is expected to be high compared to other non strictly out-crossed species. However, after the establishment of seedlings, genotypes with vigorous growth may become favoured, hence, after some time only a few genotypes should dominate the habitat due to competitive exclusion (Gary 1987). This process should then lead to a general decrease of genotypic diversity when populations are aging i.e., in later successional stages. RAPD and ISSR markers development in the plant systems are the efficient and inexpensive ways in the study of population genetics. Among all the primers tested for RAPD study, only primers having ≥60% GC content worked in our study. Similar results have been reported by Lim et al. (2000) in *N. rafflesiana* jack. This they attributed to the multiple G's or C's in the genome of *Nepenthes* species. While comparing RAPD and ISSR, most workers have found ISSR to be more informative than RAPD (Fang and Roose 1997; Esselman et al. 1999), but the reverse is also not rare. In the present study, the RAPD marker was more informative than the ISSR marker. A higher level of polymorphism was observed in RAPD (88.50%) than with ISSR (69.16%). This result was also reflected in the UPGMA cluster analysis. In RAPD, 4 groups were observed at 61% similarity whereas in ISSR, 3 diverse groups were observed at only 65% similarity. 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* Hook. f. may be because of isolation of different populations within Meghalaya. Similarly Kurata et al. (2008) reported significantly high variation in different populations of *N. vieillardii* Hook f. using 4,660 bp chloroplast DNA. They concluded that this variation is due to regional

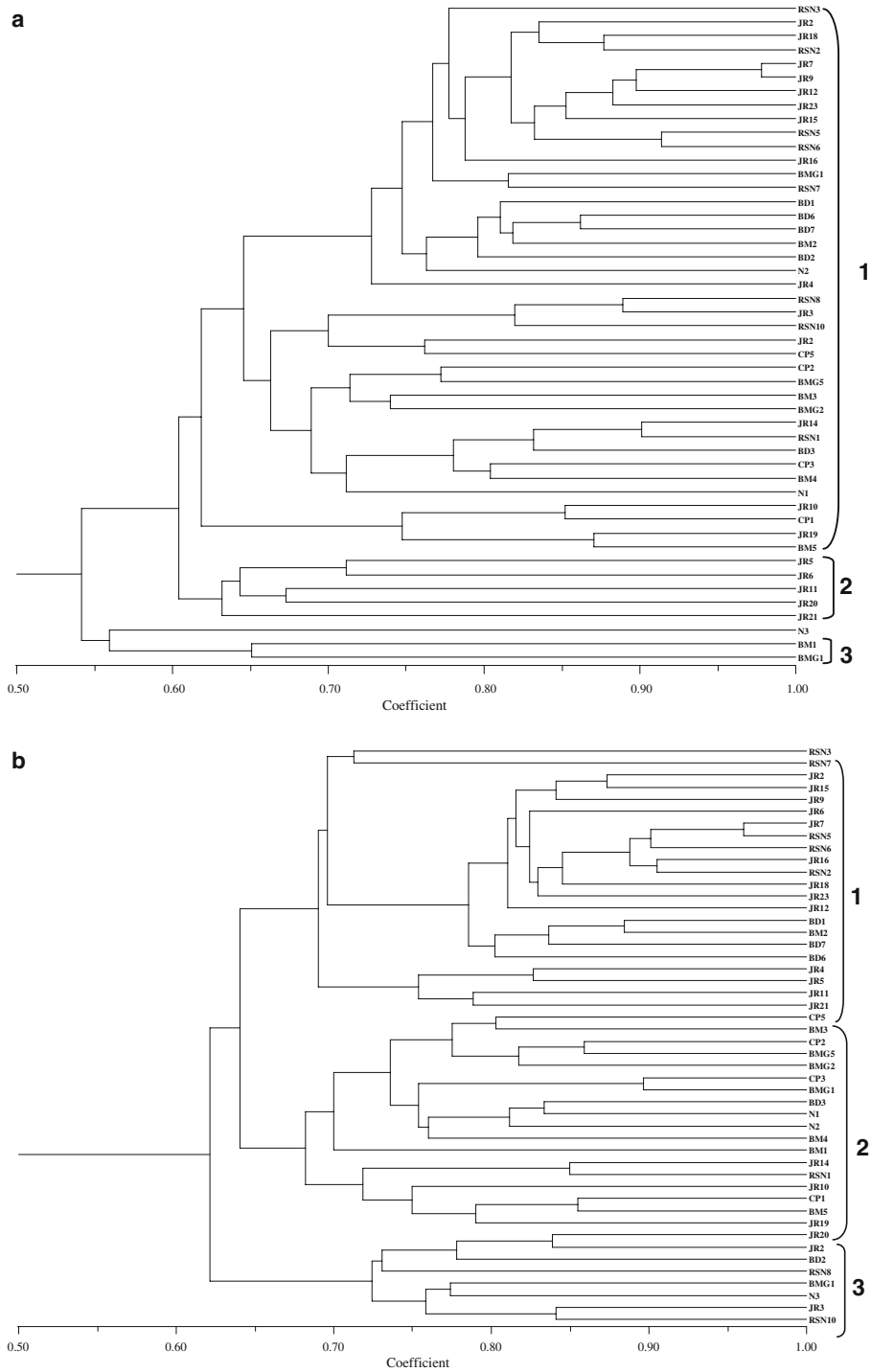

Fig. 4 **a** Dendrogram of 48 genotypes constructed from RAPD marker-based genetic similarity in *N. khasiana*. **b** Dendrogram of 48 genotypes constructed from ISSR marker-based genetic similarity in *N. khasiana*. **c** Dendrogram of 48 genotypes constructed from pooled ISSR & RAPD marker-based genetic similarity in *N. khasiana*

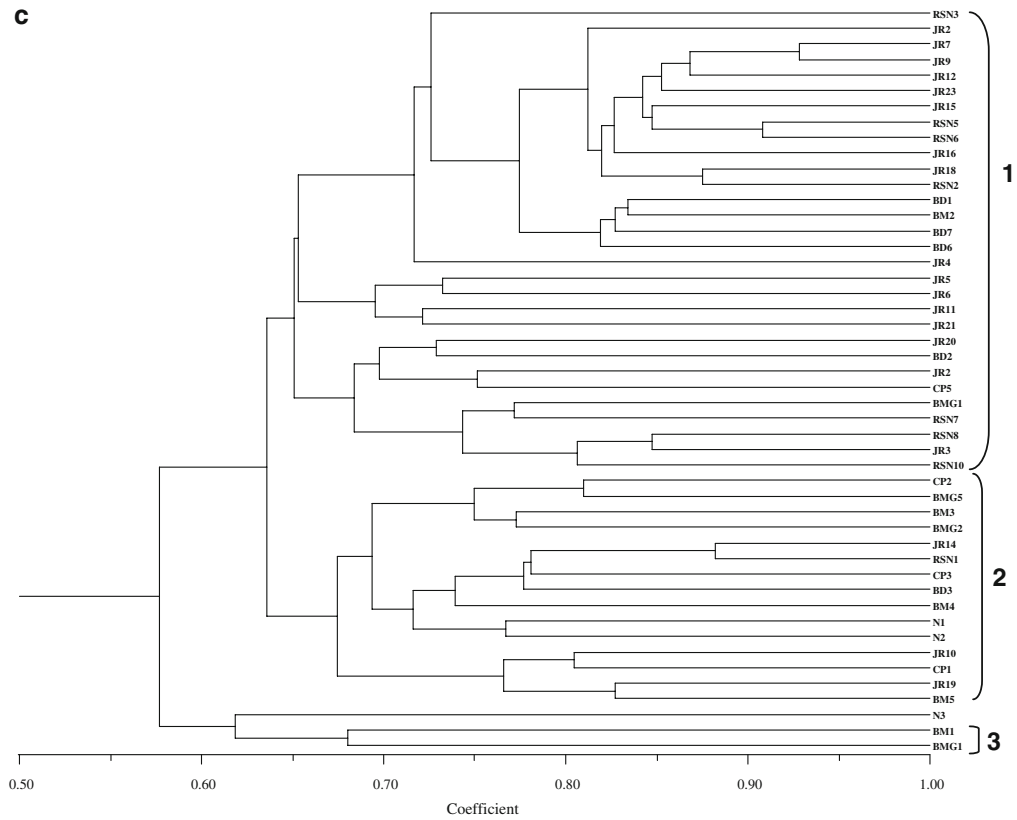

Fig. 4 continued

fragmentation resulting in the isolation of each population and significantly restricted seed flow Average Rp value for the primers was also higher in RAPD than that with ISSR. The variations detected by the markers were different because the ISSR markers amplify relatively conserved regions present among the microsatellite sequences, whereas the RAPD markers amplify random regions along the genome (Zietkiewicz et al. 1994; Fracaro et al. 2005). This was also reflected in the Mantel test. In Mantel test, although the value of correlation coefficient between RAPD and ISSR markers was significant ($r = 0.47006$) but the value of determination coefficient was very low ($r^2 = 0.2209$) which means that the two markers are differently utilizing the existing variation of the *N. khasiana* Hook. f. genome. Moreover both the markers are targeting different regions of the genome. Therefore, a comparison of data from both the marker system will provide more reliable information about the genetic variation as both the markers were utilizing a large part of the genome in two different ways. The cluster analysis based on pooled RAPD–ISSR data reveals 4 groups where groups 3 and 4 were the same as observed in RAPD. This result also reflects the usefulness of RAPD over ISSR in *N. khasiana* Hook. f. for detecting variability. The genotype N3 was coming out as a separate group while 2 genotypes from South Garo hills (BM1 and BMG1) were in another group in both RAPD and pooled RAPD–ISSR clustering. A high level of genetic diversity have been reported in some other endemic plants such as the fern *Polystichum otomasui* Kurata (Maki and Asada 1998), *Tillandsia achyrostachys* E. Morr. (González-Astorga et al. 2004), *Antirhea aromatica* Castillo-Campos et Lorence (González-Astorga and Castillo-Campos 2004) and *Sinocalycanthus chinensis* (W.·C. Cheng et S. Y. Chang) W.·C. Cheng et S. Y. Chang (Li and Jin 2006). It has been suggested that, when genetic differentiation between populations is high, large numbers of natural protected areas will be necessary for preserving the genetic diversity of a threatened

species (Frankel and Soulé 1981; Eguiarte and Pinero 1990). Loss of genetic diversity results in disturbed gene frequency in endangered species and thereby reduces evolutionary potential to adapt to changing environments (Tamaki et al. 2008). Knowledge of population structure is important for *ex situ* and *in situ* conservation of natural populations (Williams and Hamrick 1996) by maintaining the total evolutionary potential and minimizing consanguinity. If we consider the variation within populations, the population from Jarain and Rongsai had the maximum variation as the highest (JR 7 and RSN 6) and lowest (JR 2 and JR 3) similarity coefficients were observed in this population. Between the populations, the Baghmara region with NEIST exhibits about 40% dissimilarity at both RAPD and pooled RAPD–ISSR genetic similarity studies. The genotype collected from NEIST farm may have been collected from Baghmara region and planted at NEIST, Jorhat some time ago. Baghmara is the district head quarter of South Garo hills that already has a reserve for pitcher plants. High genetic diversity in the present study may be attributed mainly to long isolation of the population at different locations and to some extent due to differences in altitude, temperature, rainfall, and may be also because of the allogamous nature of the species. But whatever the cause of variation, it is necessary to conserve this variation as in fragmented environments there is a tendency to lose intrapopulation genetic variability due to the low frequency of interpopulation gene exchange (Oostermeijer et al. 2003). The inbreeding results in decreased genetic variability with increased expressions of deleterious characters, seed abortion, reduced fertilization and seed germination slowly leading the population to extinction. For conservation, the populations need to be conserved at small protected areas for proper management and allowing transfer of plants or genes from one population to another so that variability may be maintained among the populations.

Conclusion

Assessing the level and distribution of genetic diversity of rare tree species is essential for their management and the development of effective conservation strategies. These type of plants will face extinction in the near future if the causal factors of decreasing population continue operating (Khoshbakht and Hammer 2007). Our genetic diversity analysis with RAPD and ISSR provide an important contribution toward confirming that *N. khasiana* Hook. f. has well-differentiated populations in relation to their high morphological variability. The high degree of population differentiation in this species highlights the need for additional conservation measures, including measures to protect of all of the remaining populations. Haplotype diversity was detected in *N. vieillardii* by using cp DNA haplotype analysis (Kurata et al. 2008). Therefore, a thorough analysis of *N. khasiana* Hook. f. has to be done with advance techniques to know the actual cause of genetic diversity.

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