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ARTICLE

Molecular Characterization and Assessment of Genetic Similarity Among the Accessions of *Geophila repens* L. Using RAPD Markers

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Abstract

Genetic relationship was analysed among 11 different accessions of *Geophila repens* L. Plants were collected from 10 different localities in Kerala and one from Andaman islands. Polymorphism at molecular level was studied by Random Amplified Polymorphic DNA marker technique. All the eleven samples were subjected to polymersae chain reaction (PCR) using 20 arbitary decamer oligonucleotide primers. The PCR product of the plant genome gave a total of 163 bands out of which 98 were found to be polymorphic. The similarity indices ranged from 0.78 to 0.95. Both the similarity matrix and the dendrogram revealed that there is not much of genetic diversity which could be attributed to the scarcity in distribution of the species. The result suggests a need to undertake better conservation strategies to ensure its existence.

Keywords: *Geophila repens* L., RAPD, Genetic Characterization

Introduction

Geophila D. Don- repens (L) (Synonym: *Geophila herbacea* (Jacq.) K. Schum., *Rondeletia repens* L.) is a prostrate herb having medicinal properties in the family Rubiaceae. It is a small slender creeping plant rooting at nodes found in the forest areas of Western Ghats, Assam and Andaman islands. It is used in the treatment of diarrhoea, sore, earache, cough and intestinal ailments (Phytochemical database, USDA, 2005). This plant possesses properties similar to that of *Cephalis ipecacuanha*, which is used as an emetic, expectorant, antiamoebic and diaphoretic (Nadkarni, 1976). This plant is used in the traditional medicinal practices of the tribals of Thiruvananthapuram as a guarded drug to combat severe jaundice and other liver ailments. Though with great medicinal potential, Geophila is a totally under explored herb with respect to its active compounds and its action. Being restricted to specific pockets in Western Ghats forest areas and in the forest under covers in Assam and Andaman islands, the accessibility to this plant is quite difficult. Further, due to scarcity in distribution there is a threat to its existence due to over exploitation and dwindling nature of forest due to rapid urbanization and industrialization.

Assessment of genetic diversity within a plant population has important consequences in the conservation of genetic resources. The extent of genetic diversity between plant genotypes can be estimated either by determining their level of polymorphism for genetic markers or by analysis of morphological traits. Traditionally morphological characters have been used to evaluate distinctness, uniformity and stability and also to establish the description of genotype (Peterson *et al.*, 1994). Although traditional methods using morphological traits for classification are widely used it is largely unsuccessful in establishing the diversity and relationships among different species due to environmental influence on traits of interest (Raghupati and Chari, 2007). These limitations can be overcome by the advent of DNA markers, which have the advantage of being virtually unlimited and do not disturb the physiology of the organism. The PCR (polymersae chain reaction) based methods such as Random Amplified Polymorphic DNA (RAPD) are faster and cheaper compared to non-PCR based methods. Since its discovery (Williams *et al.* 1990), random amplified polymorphic DNA (RAPD) technique has been successfully employed in the assessment of genetic diversity because of their simplicity, speed and relatively low cost (Nybom, 2004). A number of scientists have used RAPD markers to study polymorphism in various plants (Ortiz *et al.* 1997, Ranade *et al.* 2002, Rout and Das, 2002, Samal *et al.* 2003, Zhang *et al.*, 2005). In the present study the purpose of investigation is to use RAPD technique to asses the level of genetic variation among accessions in *Geophila repens*.

Materials and Methods

Analysis was carried out in 11 acessions of *G. repens*, out of which 10 accessions are from different localities in Kerala and one from Andaman islands. (Table 1). Fresh young

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leaves were taken at the time of collection and stored at –20°C till extraction.

Total genomic DNA from the young leaves was isolated following the modified Murray and Thompson (1980) method using cetyltrimethylammonium bromide (CTAB). The extraction buffer consisted of 5% CTAB, 1M Tris-HCl (pH-8.0), 5M NaCl, 1% PVP, β-mercaptoethanol and 0.5M EDTA. The leaves were grinded in a mortar and pestle using liquid nitrogen and suspended in the extraction buffer. To this equal amount of chloroform, isoamyl alchohol (1:1) mixture is added and centrifuged. This process is repeated thrice followed by treatment with sodium acetate, iso propanol mixture (1:1). The precipitate is treated with 80% ethanol, centrifuged and air dried and is subjected to Rnase treatment. The material is extracted again once with phenol-chloroform-isoamylalcohol mixture, and then with chloroform- isoamyl alcohol and subsequently with sodium acatate- iso propanol mixture. DNA obtained was dissolved in TAE buffer and spectral analysis at 260nm was done to determine the concentration of DNA.

RAPD analysis was carried out using a set of 20 primers of the S series (Vision Scientific, India). PCR conditions were optimized by varying the concentrations of template DNA, primer, MgCl2 and Taq DNA Polymerase. The analysis was repeated twice to analyae the reproducibility of the bands. PCR amplification was carried out in 21µl reaction mixture containing 0.2µl dNTP's, 1.2µl $MgCl_2$, 2.5µl 10x buffer, 0.5µl Taq DNA polymerase (Bangalore Genie, India), 200pmol each of primer and 50ng of genomic DNA. Amplification was performed in a thermal cycler (Palm Cycler, Corbett, Australia). After the initial cycle of 4 min at 95°C, 40 cycles of 30sec. at 94°C, 1min at 37°C and 2min at 72°C were performed. The last cycle was followed by 5min extension at 72°C. Amplified products were electrophoresed on 1.2% agarose gel in 1X TAE buffer, stained with 1µl EtBr (ethidium bromide and analysed and photographed using the gel documentation system. Each RAPD band was treated as a character and was scored as present (1) or absent (0). The1/0 matrix was prepared for all the scored bands and data were analysed to construct similarity matrix from the binary data with Jaccards coefficients and the dendrogram was generated with unweighted pair-group method arithmetic average (UPGMA) algorithm, using SPSS. Multivariate relationships among accessions were revealed through principal component analysis (PCA) of distances.

Table 1. Accessions of *Geophila repens* collected from different localities.

Sl. No:	Accessions (Acc.)	Acc. code	Acc. No:
1	TBGRI gene bank	F	KUBH 5588
2	Kulathoopuzha	DK	KUBH5590
3	Anchal	Kd	KUBH5592
4	Mukkada	Md	3157
5	Uzhavoor	Ur	KUBH5595
6	Iringol	Il	KUBH5596
7	Thattekkad	Td	3024
8	Peechi	P	KUBH5598
9	Palakkad	D.P	KUBH5599
10	Mattanoor	Mr	KUBH5600
11	Andaman islands	An	2801

Results

In the present study 11 accessions were examined for their RAPD-PCR patterns. All the bands, both intense and faint bands were scored for calculating the similarity index values, so that a maximum number of scorable characters with minimum statistical error is obtained. All the 20 primers gave reliable amplification profile for obtaining the genetic relationships among 11 accessions of Geophila repens (Table 2). The banding profiles generated by the primers yielded a total of 163 amplification products of which 98 bands were polymorphic. Representative RAPD patterns generated by primers are shown in Figure 1.

The distance matrix generated in RAPD on the basis of Dice coefficient revealed high level of genetic similarity among the accessions though there is no 100% similarity (Table 3). The pair wise genetic similarity ranged from 0.78

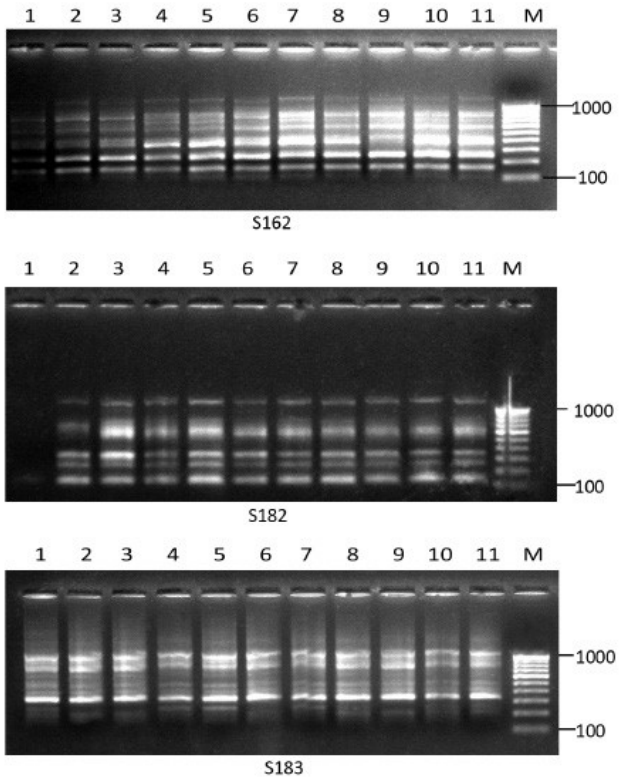

Figure 1. Representative figures of polymorphic RAPD bands generated from the genomic DNA of 11 accessions of *Geophila repens*. The lanes contains accessions in the order same as that in Table 1. 'M' is the marker

Table 2. List of 20 primers used and the polymorphism displayed by the *G. repens* accessions

Sl. No.	Primers	Total Amplicons	No. of Polymrorphic Loci	No. of Monomorphic Loci
1	S113	7	6	1
2	S105	7	0	7
3	S104	9	6	3
4	S119	8	7	1
5	S162	11	4	7
6	S150 (68.8)	6	2	4
7	S150 (50.5)	8	6	2
8	S164	6	4	2
9	S182	7	7	0
10	S166	9	8	1
11	S187	9	7	2
12	S183	11	5	6
13	S143	10	6	4
14	S142	8	6	2
15	S127	3	3	0
16	S150 ((44.3)	11	9	2
17	S193	10	1	9
18	S191	7	6	1
19	S190	8	3	5
20	S196	8	2	6
	Total	163	98	65

Table 3. Similarity matrix obtained from the RAPD data of eleven accessions of *Geophila repens* using Dice's coefficient

Accession	Accession										
	Tg	Dk	Kd	Md	Ur	Il	Td	P	Dh	Mr	An
Tg	1.00	0.86	0.83	0.78	0.80	0.83	0.81	0.81	0.81	0.81	0.79
Dk		1.00	0.83	0.83	0.85	0.88	0.86	0.84	0.86	0.84	0.84
Kd			1.00	0.85	0.87	0.89	0.87	0.86	0.88	0.87	0.84
Md				1.00	0.91	0.87	0.91	0.85	0.87	0.87	0.85
Ur					1.00	0.90	0.94	0.91	0.92	0.91	0.86
Il						1.00	0.94	0.93	0.95	0.92	0.91
Td							1.00	0.94	0.94	0.94	0.91
P								1.00	0.94	0.95	0.89
Dh									1.00	0.94	0.90
Mr										1.00	0.90
An											1.00

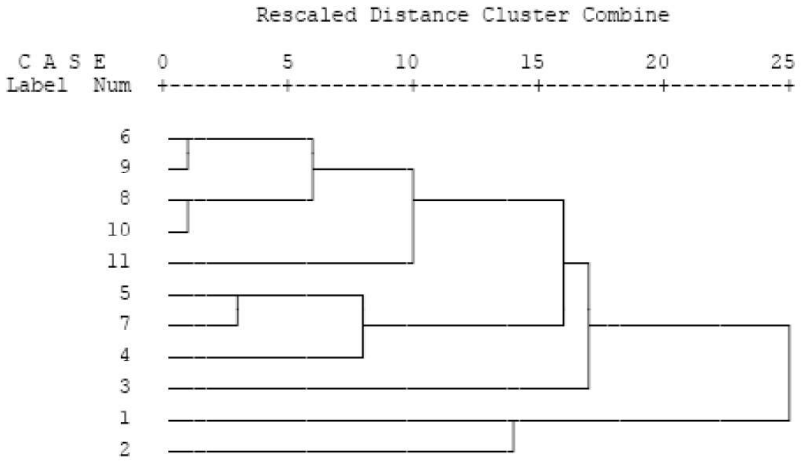

Figure 2. Dendrogram based on RAPD matrix in accessions of *Geophila repens*

to 0.95. The similarity matrix was subjected to UPGMA clustering and based on the similarity matrix a dendrogram was obtained. The cluster analysis showing the grouping between accessions is displayed in Figure 2. The dendrogram analysis indicates that the 11 accessions are grouped together into two major clusters. Cluster I is the major cluster comprising of nine accessions grouped together of which accession 3 (Kd) lies as an outlier. The position of accession 3 as a separate unit is supported by a bootstrap value of 83%. Cluster I is again separated into two sub clusters with a bootstrap value of 92% at the node. Highest value for genetic similarity (0.95) was estimated between accessions 6 (Il) and accession 9 (Dh) and between accessions 8 (P) and 10 (Mr). These accessions were seen clustered together in the dendrogram, in two subclusters respectively and their grouping was substantiated by bootstrap analysis. A higher bootstrap value of 91% was obtained at the node of clustering of accession 6 and 9. The grouping of accessions 8 and 10 into a subcluster was supported by a bootstrap value of 89%. In subcluster I, accession 7 (Td) stands apart with a bootstrap value of 92%. The other accessions in the cluster (accessions 4, 5 and 11) remain separated from each other showing significant bootstrap values (62%, 78% and 83% respectively) at their nodes. In cluster II, two accessions from nearby geographic location, i.e., accession 1 (Tg), accession 2 (Dk) are grouped together with a similarity value of 0.86. The association of accession 1 and 2 are supported by a maximum bootstrap value of 97%. Lowest estimated value (0.78) is between accession 1 (Tg) and accession 4 (Md).

Associations among the accessions were also revealed by a two dimensional PCA based on the same set of matrix. PCA does not reveal any additional grouping of samples. Overall, the grouping pattern from PCA corresponded well with the clustering pattern of the dendrogram (Fig. 3). But in PCA analysis accession 9 and accession 10 (Mr) lie very close to each other (almost overlap), though they are not so closely placed in the cluster. But they are seen in the same

Table 4. Eigen value, percentage of variability and accumulated variability for RAPD analysis

PCos	Eigen value	Percentage Variability	Cumulative percentage
1	5.734	52.129	52.129
2	1.105	10.045	62.173
3	0.88	8.001	70.175

subcluster and have a similarity index of 0.94 close to the highest similarity value. The remaining accessions are ordinated in the same way as that in the dendrogram. Accessions 3 (Kd) which is seen as an outlier in cluster I is seen lying in a separate plane. Accessions 4 (Md), 5 (Ur) and 11 (An) which stands separate in the first cluster separates out in the scatter plot also. Accessions 1 (Tg) and accession 2 (Dk) lies in the same plane in the scatter plot and are grouped together in the cluster. The percentage variability accounted by the first PC was 52.13%, 10.05% for the sec-

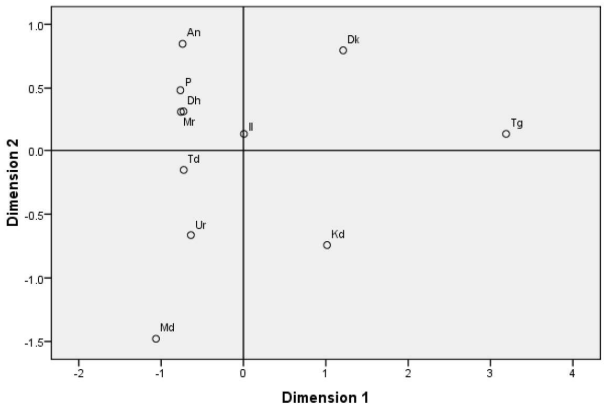

Figure 3. Principal component analysis showing the relationships among *Geophila repens* based on RAPD data

ond principle component and 8.001 for the third PC. The three components together showed a cumulative variance of 70% and the Eigen values ranged from 5.73 to 0.88. (Table 4).

Discussion

In the present study genetic characterization of 11 accessions of *G. repens* was carried out using 20 random primers. Genetic characterization of natural resources is an essential step for a better understanding of genetic resources for the implementation of in situ and ex situ conservation activities (NBPGR, 2000). Molecular data can be of great value to conservationists and it is possible to investigate directly many issues of concern such as gene pool fragmentation and genetic erosion (Falk and Holsinger, 1991). Further they are plentiful, are independent of tissue or environmental effects, and allow accession identification in the early stages of development.

DNA markers being independent of environmental interactions are highly heritable, unlimited in number and highly polymorphic, and are considered to be the best tool for estimation of genetic diversity and development of an authentic fingerprint. Such techniques reveal polymorphisms at the DNA level and are a very powerful tool for characterization as RAPD and ISSR primers can resolve the genetic diversity more precisely. The DNA fingerprinting generated by the Polymerase Chain Reaction (PCR), using arbitary primers, has provided information for estimating genetic relationships in a number of plant species (Pal and Raichaudhuri, 2003; Nayak *et al.*, 2003). DNA fingerprinting of all the genetic resources of the medicinal plants is a necessity for generating a molecular database as well as to utilize the information in a systematic manner. With this as a premise, given that a plant's morphology arises through interactions between genes and environment, it makes all the more imperative to use molecular markers to catalogue *Geophila repens* accessions based on the geographical variations.

The random amplified polymorphic DNA technology (Williams *et al.*, 1990) is the most appropriate and convenient technique in genotype fingerprinting (Kumar *et al.*, 2006). Being multilocus (Karp *et al.*, 1997) it is the simplest and fastest detection technology for diversity analysis. In the present study RAPD analysis revealed low level of genetic variation among the accessions of *G. repens.* RAPD profiling using 20 primers revealed polymorphism of about 60%. In this analysis only two primers gave full polymorphism and a single primer was found to be monomorphic. Similarity matrix obtained using Dice coefficient gave pair wise genetic similarity values ranging from 0.95 to 0.78. Dendrogram obtained using these similarity values, showed greater similarity between accession 6 and accession 9 and also between accessions 8 and 10 as depicted by the matrix. The robustness of the dendrogram was substantiated by high bootstrap values.

The similarity values obtained with the RAPD data were higher, which was also evident from the gel. Almost similar pattern of bands were obtained among the accessions in the gel when RAPD was done using 20 arbitrary primers. The result is on par with the RAPD analysis of *Typhonium sp.* by Rout (2006) and in Ixora sp. (Rajaseger *et al.*, 1997). The clustering of accessions in the dendrogram based on the similarity matrix is supported by a PCA scatter plot. Results obtained from the principal component analysis correspond well with the grouping of accessions based on cluster analysis with the exception of minute rearrangements. Analysis using RAPD revealed similarity among accessions which was evident from the similarity matrix and PCA analysis. These similarities measure the relative genetic relatedness among the accessions within a group. A higher similarity would mean more genetic relatedness among the accessions within the group. Three principal components obtained accounted for 70% of the total RAPD variation (52.13%, 10.05% and 8.001, respectively). Though these components explained higher cumulative variation, the results of the PCA were generally consistent with those obtained through the clustering analyses.

RAPD technique is advantageous as it can yield a large number of loci and may provide a more representative sample of the genome than proteins and allozymes do. RAPD markers are even more abundant because numerous random sequences can be used for primer construction. Genomic abundance is essential to studies where a large fraction of the genome needs to be covered, e.g. for the development of high-density linkage maps in gene mapping studies. However, the RAPD technique has also some limitations. The most important one is the dominant allelic expression, which will bias the estimates of genetic diversity and population genetic structure (Lynch and Milligan, 1994; Isabel *et al.*, 1993; Szmidt *et al.*, 1996).

In the present study RAPD analysis revealed low level of genetic variation among the accessions of *G. repens.* Genetic variation is vitally important to enable a species to evolve and adapt in a particular environment thereby ensuring the stability of a forest ecosystem. The low level of variation among the accessions revealed by RAPD primers suggests that *G. repens* has a narrow genetic base. The fragmented distribution may be due to lack of genetic diversity, which might be rendering it less adaptable to varied environmental conditions. Rare plant species are commonly hypothesized to have little genetic variation because of genetic drift, strong and directional selection towards genetic uniformity in a limited number of environments, inbreeding depression or other factors (Lee *et al.* 2003). According to Karron (1991), most of the rare plant species reviewed revealed low to moderate levels of genetic diversity.

Conclusion

In the present study, genetic variation was analysed in *G. repens*, a rare medicinal plant by examining 168 amplicons in 11 accessions. The study was also carried out to analyse the

genetic reason for the restricted distribution of the plant. The study revealed very low level of genetic diversity among the accessions and this could be attributed to the range fragmentation of the species. When diversity is less population distribution is also less and hence better conservational strategies have to be taken to ensure its conservation. For better understanding of this issue further studies are needed in near future using highly variable molecular markers like AFLP.

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