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Genetic Diversity Analysis of *Celastrus paniculatus* Willd. – a Nearly Threatened, Cognitive and Intelligence Enhancer – by RAPD Markers

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ABSTRACT

Celastrus paniculatus (Celastraceae) is a Red listed medicinal plant and the species has been overexploited over the last few decades for its medicinal uses. Information on its genetic diversity is currently lacking for strengthening its conservation management practices. Random amplified polymorphic DNA (RAPD) polymorphism was applied to check the efficiency of *ex situ* genetic conservation. Using this RAPD analysis, we observed a significant genetic variation among the collected accessions of *C. paniculatus*. Modified CTAB protocol yields a high quality DNA and the annealing temperature was standardized at 37°C for RAPDPCR reactions. Fourteen random primers, each with ten base pairs generated 143 bands with 91% being polymorphic, which were used to estimate genetic distances among the collected accessions. The species show higher genetic diversity between accessions than other red listed medicinal plants. Mean locus similarity among individuals was 0.721 for all pairwise comparisons. The present work provides important baseline data for conservation and collection strategies for this species. Thus RAPD markers proved to be useful in distinguish variation in very rare species which are of conservation concern.

Keywords: accession specific markers, conservation, genetic diversity, polymorphism, Random amplified polymorphic DNA, Red listed medicinal plant

INTRODUCTION

Celastrus paniculatus Willd. (Celastraceae) is a scandent shrub distributed up to at an altitude of 1800 m throughout most parts, chiefly in deciduous, semi evergreen and evergreen forests of India (Parrotta 2001). Though the species were previously well established, now it has come under the red listed category as 'nearly threatened' due to over harvesting and indiscriminate exploitation from the wild (Anonymous 2000). It is popularly called as "Cognitive and Intelligence enhancer". Different plant parts are also used as various curative medicines. The seeds are used to increase intelligence and memory power (Khare 2004). Pharmacological studies suggest that the seed oil obtained from the seeds possesses sedative and anticonvulsant properties and a number of additional pharmacological actions such as analgesic (Ahmad *et al*. 1994), hypolipidemic (Khanna 1991), antispermatogenic (Wangoo and Bidwai 1988), anti-inflammatory (Dabral and Sharma 1983), insecticidal (Atal *et al*. 1978) and anti-bacterial (Patel and Trivedi 1962). Seed oil is also shown beneficial effects in treating psychiatric patients and improving certain psychological attributes, including IQ in mentally retarded children (Nalini *et al*. 1986).

The assessment of genetic diversity in natural populations can provide a new approach into the evolutionary history and phylogenetic relationships of *C. paniculatus*. In addition, the development of appropriate strategies for the conservation of plant genetic resources requires a detailed knowledge of the amount and distribution of genetic variation within the species (Vaughan 1994). Such information can be obtained using biochemical and molecular methodologies. However, molecular techniques have several advantages over biochemical methodologies such as speed, low cost, and the use of small amounts of plant material (Heun 1994). One of these techniques has been called RAPD (Random Amplification of Polymorphic DNA). In this method, short oligonucleotides of arbitrary sequence are used singly to support the amplification of regions of the test plant genome and the amplification products are separated by gel electrophoresis; differences between genotypes are reflected as differences in the banding patterns (Williams *et al*. 1990; Hedrick 1992). In recent years RAPD analysis has become a popular method for estimating genetic diversity analysis within germplasm collections of several medicinal plants, for example *Andrographis paniculata* (Padmesh *et al*. 1999), *Santalum album* (Shashidhara 2003) and *Azadirachta indica* (Deshwal 2005). Although earlier studies have reported some results of research on *in vitro* propagation, cytological and chemical analysis of *C. paniculatus*, no information was given on genetic diversity (Zhang *et al*. 1998; Nair and Seeni 2001; Sharada 2003). The purpose of the present study is to assess the extent and distribution of genetic variation among the collected natural populations of *C. paniculatus*, using RAPD markers which will provide a theoretical basis for conservation without further extinction.

MATERIALS AND METHODS

Population sampling

Total DNA was extracted from the ten accessions of *C. paniculatus* plants grown in a field gene bank, collected from different localities of Andhra Pradesh state representing eco-geographical diversity (**Table 1**). The distance between the collected accessions was at least 40-50 km to increase the possibility of detecting the variation potential within each population, according to Fu *et al*. (2003). Leaves were dried in plastic bags with silica gel until extracted in the laboratory. A total of 40 primers were screened using pooled samples of DNA from ten accessions.

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Table 1 Germplasm accessions of *Celastrus paniculatus* investigated for genetic diversity analysis.

Accession name	Location	District	Latitude	Longitude
Cp Gb	Gachibowli	Ranga Reddy	17° 38′ N	78° 12′ E
Cp Tk	Talakona	Chittoor	13° 71′ N	79° 33′ E
Cp Sr	Srisailam	Kurnool	16° 03′ N	78° 34′ E
Cp Gd	Gogarbhamdam	Chittoor	13° 71′ N	79° 40′ E
Cp Tr	Taliperu	Khammam	18° 11′ N	80° 82′ E
Cp Sk	Sukumamidi	Khammam	17° 74′ N	81° 63′ E
Cp Pk	Pakhala	Warangal	17° 84′ N	80° 12′ E
Cp Bp	Balpally	Cuddapah	14° 18′ N	78° 74′ E
Cp Mr	Mallur	Warangal	18° 24′ N	80° 30′ E
Cp Vd	Vikarabad	Ranga Reddy	17° 51′ N	77° 44′ E

Isolation of DNA

Genomic DNA was extracted from leaves of specific individual accession based on the CTAB (hexadecyl trimethyl ammonium bromide) (Sigma Aldrich, USA, Molecular Biology tested) method developed by Doyle and Doyle (1987) with slight modifications, as this method is successfully used on a broad range of tissues, fresh or dried, including calli from many species. Leaf material (1 g) was powdered in liquid nitrogen along with 0.05 g of PVPP (Sigma Aldrich, USA, Molecular Biology tested) (polyvinylpolypyrrolidone), mixed with preheated 2% CTAB (5 ml) extraction buffer (2% CTAB, 1.4 M NaCl (Qualigens fine chemicals, GSK, India, analytical grade) 100 mM Tris HCl (Qualigens fine chemicals, GSK, India, analytical grade), pH 8, 20 mM EDTA (Qualigens fine chemicals, GSK, India, analytical grade)), and the homogenate was incubated at 65°C for 90 min in a water bath. Equal volumes of chloroform:isoamylalcohol (24:1) (Qualigens fine chemicals, GSK, India, analytical grade) was added to the extract prior to centrifugation at 12,000 rpm for 15 min to remove the proteins. To the supernatant equal volumes of ice cold isopropanol (Qualigens fine chemicals, GSK, India, analytical grade) was added and incubated at –20°C for a period of 30 min followed by centrifugation at 12,000 rpm for 15 min. The pellet was washed with 70% ethanol (Qualigens fine chemicals, GSK, India, analytical grade), centrifuged at 8000 rpm for 8 min and was dried and dissolved in 30 µl of TE buffer (10 mM Tris HCl, pH 7.5, 1 mM EDTA). In order to eliminate RNA, the sample was treated with 2 µl RNase A (10 µg/ml) and the solution was incubated overnight at 37°C. This was followed by phenol (Amersham Biochemicals, UK, molecular grade): chloroform:isoamylalcohol (25:24:1) extraction followed by centrifugation at 8000 rpm for 15 min. To the supernatant equal volumes of chloroform:isoamylalcohol (24:1) was added and centrifuged at 10,000 rpm for 12 min. To the supernatant $1/10^{th}$ volume of 3 M sodium acetate (Qualigens fine chemicals, GSK, India, analytical grade) and equal volumes of ice-cold isopropanol was added and left for 30 min to precipitate DNA followed by centrifugation at 12,000 rpm for 15 min. The sediment was washed with 70% ethanol and centrifuged at 8000 rpm for 8 min at 4°C. After complete drying the pellet was dissolved in 50-100 µl of Tris-EDTA buffer (volume depending on the size of the pellet) and DNA samples were stored at –20°C.

Quantification of DNA

The most widespread methods for quantifying DNA are: (i) the comparison of an aliquot of the extracted sample with standard DNAs like *Hind*III digested λ DNA, of known concentration using gel electrophoresis and (ii) spectrophotometric determination at an absorbance ratio of A_{260}:A_{280}. With both methods additional information is gained concerning the quality and purity of the extracted sample obtained which is useful in carrying out different techniques such as PCR and RAPDs. DNA isolated from leaf tissues was quantified based on the spectrophotometer measurements of UV absorption at A_{260} and A_{280}. In a pure DNA sample this ratio should be around 1.8. Lower OD values indicate protein or phenol contamination and higher values indicate RNA contamination. Based on the OD values obtained, an A_{260} of 1 corresponds to approximately to 50 ng of DNA and the concentrated DNA was diluted with ultrapure Milli Q (Milli Q academic) sterile water to 50 ng/µl for PCR reactions (Sambrook 1989). DNA samples were analyzed by gel electrophoresis on 0.8% agarose in TBE buffer. Gels were stained with ethidium bromide and visualized on a UV transilluminator, photographed with a gel documentation system (LTF Labor Technik, Germany).

RAPD analysis

Forty different decamer random primers (Primer kit OPA and kit OPC, Operon Technologies, Alameda, CA) with 10-bp oligonucleotides of random sequence were screened among ten accessions. RAPD reactions were carried out in a DNA Thermocycler (MJ Research Inc., USA) with a final reaction volume of 15 µl which contained about 50 ng of template DNA, 1x PCR Buffer (10 mM Tris HCl pH 5.3, 50 mM KCl), 3 mM $MgCl_2$, 0.2 mM dNTP Mix, 0.5 µM of single primer and 1U of *Taq* DNA *polymerase*. The programme commenced with a initial denaturation step of 3 min at 94°C, followed by 30 cycles of 45 sec at 94°C, 1 min at 37°C, extension was carried out at 72°C for 1 min and final extension at 72°C for 7 min and a holding temperature of 4°C at the end. All the PCR reactions were repeated at least twice to ascertain the reproducibility of the bands. The amplification products were electrophoresed in a 2% (w/v) agarose gels stained with ethidium bromide (0.5 µg/ml) in TBE buffer (89 mM Tris, pH 8.0, 89 mM boric acid, 2 mM EDTA) at 50-60 V for 2-3 hrs. Gels with amplification fragments were visualized and photographed under UV light. Lambda DNA *Eco*R1 *Hind*III double digest was used as molecular marker in the gels as a size reference.

Data analysis

For each accession, polymorphism was scored as 1 for the presence and 0 for absence of a band. Ambiguous bands that could not be clearly distinguished were not scored. Only the clearest and strongest bands were used for cluster analysis. RAPD data generated with primers were used to compile a binary matrix for cluster analysis using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, Biostatistics, New York, USA, software version 2.02j) package. Genetic similarity among accessions was calculated according to Dice similarity coefficient (Dice 1945). The similarity coefficients were then used to construct a dendrogram using the UPGMA (Unweighted Pair-Group Method with Arithmetical averages) through Numerical Taxonomy and Multivariate Analysis System program package for PC (NTSYS-pc, version 2.0) (Rohlf 1998).

RESULTS

The modified CTAB protocol of DNA isolation from ten different accessions showed high molecular weight band without smearing, indicating that it is largely intact without any fragmentation. The spectrophotometric results (A_{260}: A_{280}) of 10 samples of DNA was between 1.2 and 1.4, shows the purity of DNA by this protocol. One g of leaf tissue produced sufficient quantity (40-100 µl) of DNA from each of the ten individuals for PCR reactions using the 10mer oligonucleotides listed in **Table 2**. DNA was diluted to 50 ng/µl with sterile water, for RAPD reactions.

Optimization of RAPD protocol

A total of 40 primers were screened among the 10 accessions of *C. paniculatus*. To optimize the RAPD assay several parameters like concentration of magnesium ions, template DNA, primer concentration, duration of time during the denaturation stage, annealing temperature of the amplification cycle and varied quantities of polymerase enzyme were examined as described by Caetano-Anolles (1993). In order to optimize annealing temperatures, the reactions were tested at 35°C, 37°C, 39°C and 42°C. The decamer primers were clearly amplified at 37°C. In addition to the above reported factors certain primers produced reliable banding patterns only when used at a higher concentration (>0.5 µM) as mentioned in the above protocol. Under the optimized conditions described in the material and methods, 14

Table 2 Information regarding code, sequence of random primers and percentage of polymorphism among the investigated 10 accessions of *Celastrus paniculatus*.

Primer Code	Primer sequence 5′–3′	Total bands	Polymorphic bands	% of polymorphism
OPA-01	CAGGCCCTTC	12	10	83.3
OPA-02	TGCCGAGCTG	10	10	100
OPA-03	AGTCAGCCAC	9	8	88.8
OPA-04	AATCGGGCTG	7	7	100
OPA-05	AGGGGTCTTG	12	11	91.6
OPA-06	GGTCCCTGAC	8	8	100
OPA-09	GGGTAACGCC	8	7	87.5
OPA-10	GTGATCGCAG	15	12	80.0
OPA-14	TCTGTGCTGG	6	6	100
OPA-15	TTCCGAACCC	10	9	90.0
OPA-17	GACCGCTTGT	6	6	100
OPA-18	AGGTGACCGT	14	14	100
OPA-20	GTTGCGATCC	9	9	100
OPC-05	GATGACCGCC	17	10	58.8

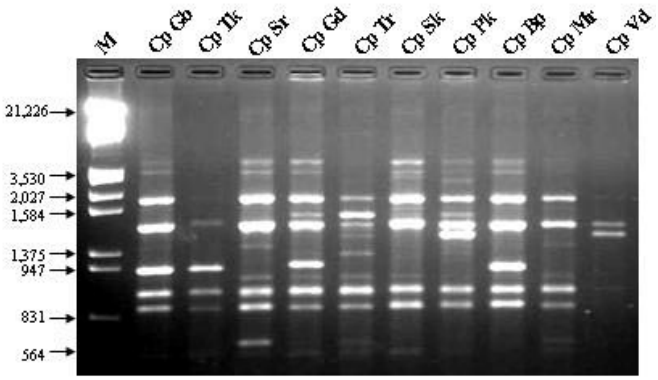

Fig. 1 Agarose gel showing polymorphic bands obtained using arbitrary primer OPA-10 5′-GTGATCGCAG-3′ from 10 different accessions of *Celastrus paniculatus* genomic DNA. M, molecular marker, λ DNA *Hind*III *Eco*RI double digest.

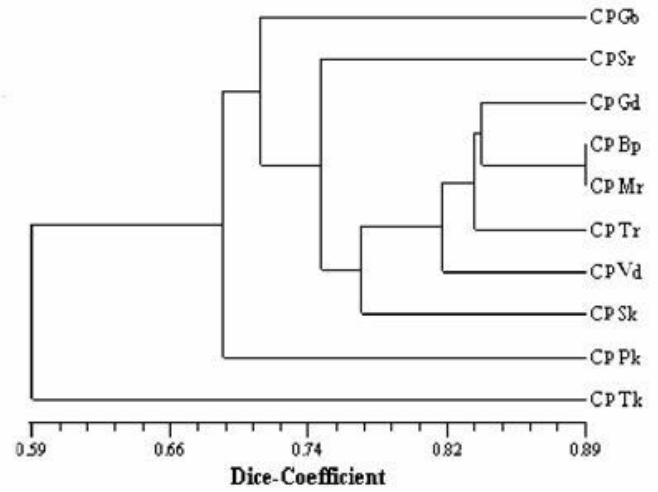

Fig. 2 UPGMA dendrogram of a RAPD based data representing genetic diversity relationships among ten randomly chosen accessions of *Celastrus paniculatus*.

primers out of 40 generated RAPD fragments based on consistent production of strong amplification products and production of uniform, reproducible fragments between replicate PCRs. A gel showing typical amplification products with OPA-10 primer is shown in **Fig. 1**. The total of 14 primers yielded 143 reproducible bands of which 16 were consistent and 127 were polymorphic bands giving a mean of 9 polymorphic bands per primer (**Table 2**). The number of polymorphic bands varied with different primers. Bands amplified ranged in size from 600 base pairs (bp) to 3500 (base pairs), although most ranged between 800 bp and 3000 bp in length. Overall 91% of polymorphism was observed among the 10 accessions of *C. paniculatus*. Mean locus similarity among individuals was 0.721 (range 0.496-0.892) for all pair wise comparisons. The minimum similarity of 0.496 was exhibited by Srisailam (Kurnool Dist.) and Talakona (Chittor Dist.) accessions, where as maximum similarity of 0.892 was exhibited by Mallur (Warangal Dist.) and Balpally (Cuddapah Dist.) accessions (**Table 3**). Clustering of localities only occurred when the average distance between clusters was high, indicating large differentces between localities (**Fig. 2**). In cluster diagram two main groups were observed with a relative distance of 0.7, showing Talakona accession is more distant from all other accessions. Remaining 9 accessions clustered at various levels irrespective of the geographical similarity or distance. Average clustering of regions indicated that the closest affinities occur between the Balpally and Mallur accessions (exhibited a genetic identity of 0.89), which link with Taliperu, Vikarabad and Sukumamidi accessions to form a well defined cluster. The most geographically closer localities of Talakona and Gogarbhamdam (a relative distance of 25 km) accessions found in separate groups and showed that they are genetically distant. Among the responding 14 primers, OPC-5 produced maximum number of bands (17), while OPA-14 and OPA-17 equally produced minimum number of bands (6).

DISCUSSION

This research was done to characterize the extent of genetic variation in ten accessions of *C. paniculatus* found in Andhra Pradesh State, using RAPD markers. For large-scale RAPD analyses, it is desirable to use a method for DNA isolation that allows fast extraction of DNA from a large number of samples while ensuring uniformity of yield and purity of samples and minimizing the risk of cross contamination. The modified CTAB method followed here resulted, a good yield with a range of 50-100 µg/g of young leaf tissue. DNA from young, uninfected tissue provided the most consistent results since tissue age and pathogen infestation may introduce potential sources of error in RAPD analysis according to Staub *et al*. (1996). The problem often encountered in RAPD analysis is that of reproducibility of band patterns between different PCR reactions. This aspect can be overcome by using a thoroughly optimized PCR protocol and by scoring only reproducible bands (Munthali *et al*. 1992). Reproducibility is sometimes difficult due to mismatches occurring between the primer and its target sequence in the amplification reaction. In fact different thermocyclers, temperature profiles, the brand of DNA polymerase, and the concentration of $MgCl_2$, primer and template DNA can affect the reproducebility of RAPD assay. Thus a standard methodology should be devised for RAPD assay to obtain identical RAPD pattern as described by MacPherson *et al*. (1993). However, in our experiments the annealing temperature was standardized at 37°C, which results in continuous high reproducibility of bands. In addition to this, certain primers produce more reliable banding pattern when used in higher concentration; this probably due to the genomic DNA used possesses an unusually high frequency of annealing sites for these primers so that their effective concentration for amplification is lowered in the reaction volume (Virk *et al*. 1995).

RAPD analysis, when used to study genetic polymerphism in a germplasm collection has an advantage over other methods due to its simplicity and rapidity. RAPD assessment of ten populations of *C. paniculatus* indicates a high level of genetic variation with 91% of bands being polymorphic and an average of nine polymorphic amplification products or loci, presumably because of the different accessions were examined. The higher genetic diversity of *C. paniculatus* populations confirmed by the UPGMA tree topology, may be explained by high levels of outcrossing rates which may also have contributed to the findings in the present study. Because of the high rates of cross pollination, gene flow and high fecundity may be a factor that led to

Table 3 Similarity matrix of *Celastrus paniculatus* generated from Dice estimate of similarity based on the number of shared fragments.

	CpGb	CpTk	CpSr	CpGd	CpTr	CpSk	CpPk	CpBp	CpMr	CpVd
CpGb	1.000									
CpTk	0.617	1.000								
CpSr	0.720	0.496	1.000							
CpGd	0.784	0.609	0.773	1.000						
CpTr	0.698	0.644	0.715	0.834	1.000					
CpSk	0.650	0.517	0.705	0.797	0.793	1.000				
CpPk	0.666	0.632	0.625	0.678	0.791	0.670	1.000			
CpBp	0.769	0.618	0.815	0.844	0.838	0.761	0.736	1 .000		
CpMr	0.710	0.563	0.784	0.826	0.820	0.784	0.703	0.892	1.000	
CpVd	0.666	0.601	0.687	0.761	0.826	0.705	0.674	0.837	0.829	1.000

higher genetic diversity with in these populations (Slatkin 1987). The high level of variation found among accessions suggests that sampling from a few localities for conservation could capture a large proportion of the variation within the species (Runo 2004). The accessions collected from Gogarbhamdam, Balpally, Mallur, Taliperu, Vikarabad and Sukumamidi formed a cluster that excluded Srisailam whereas the remaining four accessions, for which the gene flow might have been different, resulted in their separation and grouping into a new cluster. This result probably is due to the high number of monomorphic RAPD fragments and can be attributed to genetic drift. Genetic drift over thousands of generations would lead to significant divergence. This trend may be reinforced for adaptive traits by selection of important ecological differences existing among the areas from where the accessions have been sampled (Ellstrand and Elam 1993). Results from RAPD analysis indicates that genetic drift might have occurred among the studied accessions of *C. paniculatus*, thereby producing differentiation. This may be also due to highly cross-pollinated nature of the plant and due to the occurrence of mutations in the genome that may represent entirely a different variant. This wide range of variation may also be due to pollen flow and local selection (environmental) pressures.

Generally, diversity within accessions collected in a state was low, but here differences due to climatic, geographical and edaphic factors high genetic diversity have been noticed with decreasing population. The unexpected, relatively high levels of genetic variation in *C. paniculatus* make it a suitable candidate for conservation management, protection or even relocation. Accurate estimates of diversity are a prerequisite for optimizing sampling strategies and for conservation of plant genetic resources. The relatively high genetic diversity obtained seems due to large phylogenetic distance among the collected accessions. This variability maybe due to ingestion and dispersal of seeds by animals in addition to the effect of ecological, biotic and abiotic factors (Parsons 1999). The main reason for the gradual decrease in the size of the population was incessant human activity and overexploitation, along with environmental changes in their natural habitat. Otherwise with a larger area of population, the probability of crossing among individuals increases, which results in the retention of genetic variation (Ellstrand and Elam 1993). The high rate of variation observed among ten accessions with different RAPD primers means that a high number of primers must be tested in order to obtain a conclusion. Similarly the number of accessions in this study is, however, too low for a definite conclusion. More accessions are needed to conclude about the relatedness among accessions. These studies indicate that RAPDs are sufficiently informative and powerful to assess genetic variability among natural populations of *C. paniculatus*. Thus RAPD markers will provide a useful tool in the future design of collection strategies for germplasm conservation. The relatively small numbers of plants examined in this study are a direct reflection of the rarity of the genus. Our results also demonstrate the utility of RAPD markers for detecting variation in very rare species. These are rare endemics are of concern for conservation, and RAPD loci may prove useful for preservation when other methods may fail to detect variation. The potential of molecular markers have also been shown in other plants for characterization and identification of core collection for germplasm conservation (Ayad *et al*. 1997). The high levels of genetic variation measured in *C. paniculatus* despite their small populations, supports the proposal of Tansley (1998) that the use of small nature reserves is viable for the conservation of a species. The unique segments of DNA, which are often variable in size between different accessions produced with few primers, can be termed as accession specific markers and can serve as polymorphic genetic markers. Though allozyme studies were also used to detect genetic variation among different species, here PCR based markers (RAPD markers) have been employed as an alternative and are more variable than the allozyme markers which provide an important contribution toward confirming that *C. paniculatus* has well differentiated populations, despite their low morphological low variability (Ayres and Rayan 1997).

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