



# Molecular fingerprinting of the Indian medicinal plant *Strychnos minor* Dennst

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## ABSTRACT

Genomic DNA of *Strychnos minor* (Dennst) of sixteen geographical regions were amplified using thirteen primers. The analyses of products of Random Amplified Polymorphic DNA (RAPD) revealed polymorphism among the samples. The polymorphic information content (PIC) was maximum for the primer of OPB-04 (0.40) followed by the primer OPB-20 (0.39). Clustering analysis by Jaccard's coefficient index exhibited similarity matrix and distance matrix. The amplified products of the 13 primers collectively showed similarity index ranged from 0.654 to 0.986. The phylogenetic trees were constructed by UPGMA cluster analysis. Significant variations in the amplified genomic DNA suggest genetic variability of population but low inter-population genetic segregation.

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## 1. Introduction

Molecular fingerprinting which have been frequently used for studying genetic diversity, population genetics and genetic characterization in various plant species and crop cultivars. The molecular markers are not influenced by the external environmental factors unlike that the morphological markers hence accurately detect the genetic relationship between among the plant species [1]. Recently, it was determined about that the 35% of the plant species that the standing vegetation are vulnerable to elimination because they are not represented in the seed bank of the Red Sea area [2]. Proper identification is need for the closely related taxon of the botanicals [3]. DNA based markers for the authentication and identification of medicinal plant importance of DNA fingerprinting for the medicinal herbs [4]. DNA fingerprints led to the identification of closely related plant species. DNA is most stable and does not vary seasonally and with the age of plant species [5]. Many of the studies have found out the effects of genetic diversity and population of plant species [6]. *Strychnos minor* (Dennst.) (Synonym: *Strychnos colubrina*) belongs to the family Loganiaceae. It is a woody climber (Liana) found in Tamil Nadu and Kerala states of Southern India [7]. It's commonly known as snake-wood, which have high medicinal importance [8].

Among the polymerase chain reaction (PCR)-based molecular techniques, randomly amplified polymorphic DNA (RAPD) is convenient in performance and does not require any information about the DNA sequence to be amplified [9]. Among the polymerase chain reaction (PCR) based molecular techniques, random amplified polymorphic DNA is convenient in performance and does not need any information about the DNA sequence to be amplified [10]. RAPD has been used for estimate genetic diversity in various plant species [11]. The conservation need for geographical protection needs clear characterization based on biochemical or molecular fingerprinting, besides geographical specialization to classify it exactly [12]. Recently, RAPD has been used for the estimation of genetic diversity in different endangered medicinal plant species [13]. In this current study, we successfully utilized the RAPD techniques for rapid characterization of 16 different localities of Coromandel Coast of Tamil Nadu.

## 2. Material and methods

### 2.1. Plant material

Young leaves of *S. minor* were collected from the different locality of Coromandel Coast of Tamil Nadu (Fig.1). The plant materials were separately placed in sealable polythene bags and authenticated. The samples were transported to the laboratory and then stored separately in the deep freezer at  $-20^{\circ}\text{C}$  for until for DNA extraction. A complete explanation of accessions listed in (Table 1).

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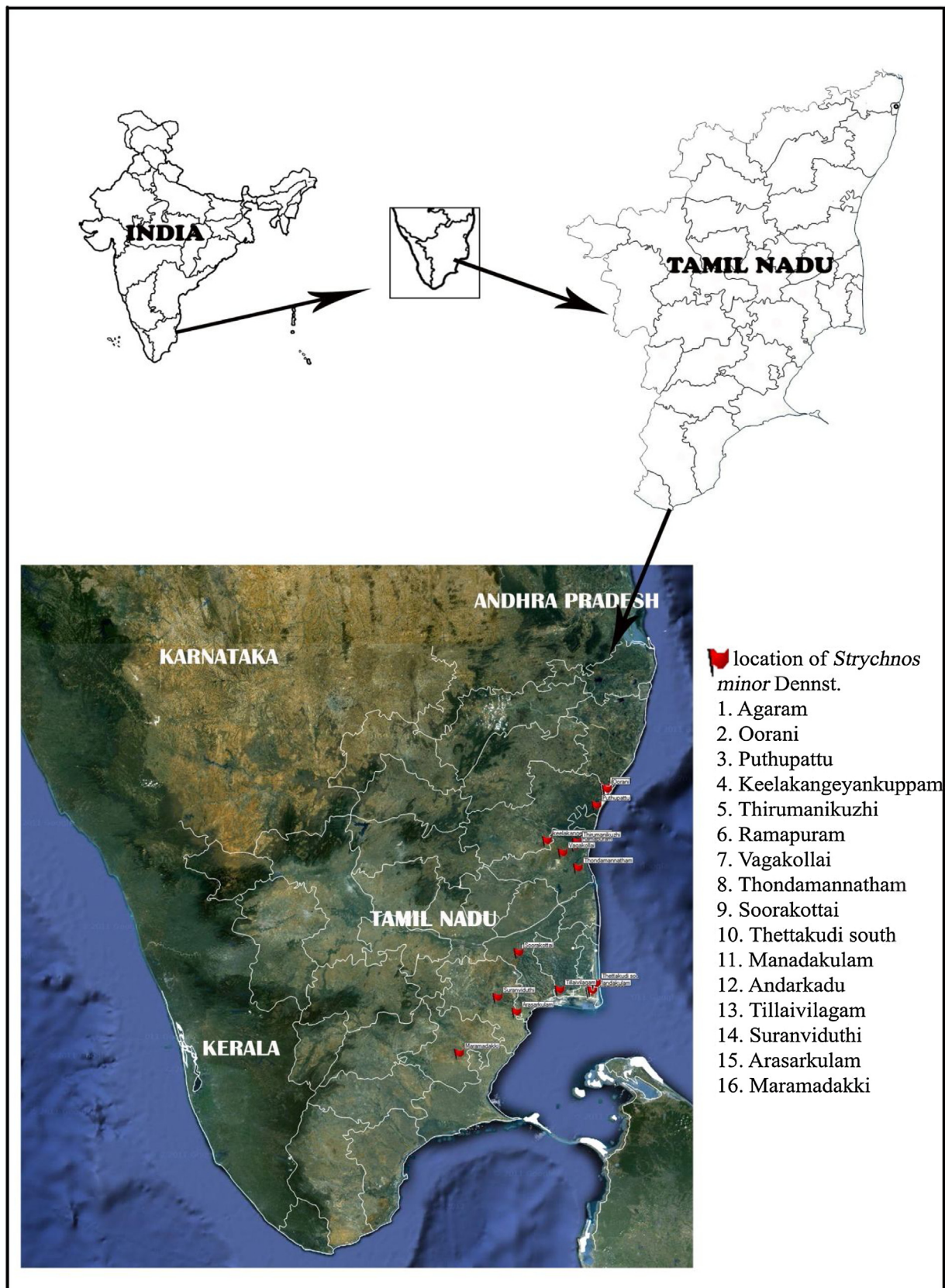


Fig. 1. Geographical distribution of *Strychnos minor* collected from Coromandel east coasts of Tamil Nadu, India.

**Table 1**Geographical location of sample site for each locality of *S. minor*.

District	Location	Latitude and longitude
Villupuram	Agaram	12°12'N79°56'E
	Oorani	12°11'N79°57'E
	Puthupattu	12°03'N79°52'E
Cuddalore	Thirumanikuzhi	11°43'N79°41'E
	Ramapuram	11°41'N79°41'E
	Thondamannatham	11°29'N79°42'E
	Keelakangeyankuppam	11°44'N79°26'E
	Vagakollai	11°37'N79°34'E
Pudukottai	Maramadakki	09°50'N78°40'E
	Arasarkulam	10°12'N79°10'E
	Suranviduthi	10°20'N79°00'E
Nagapattinam	Manadakulam	10°24'N79°49'E
	Andarkadu	10°23'N79°48'E
	Thettakudi south	10°27'N79°51'E
Thiruvavur	Tillaivilagam	10°24'N79°32'E
Tanjavur	Soorakottai	09°50'N79°25'E

## 2.2. Chemical and Reagents

An extracting buffer containing 3% CTAB (w/v), Tris HCl pH 8.0 (1.0 M); EDTA pH 8.0 (0.5 M); NaCl (1.4 M), 2% PVP (w/v) and 0.2% mercaptoethanol was prepared. Ribonuclease A (10 µg/ml), Chloroform: Isoamylalcohol (24:1), Phenol:Chloroform:Isoamylalcohol (25:24:1 v/v/v), TE buffer (Tris HCl 100 mM, EDTA 20 mM, pH 8.0) and Ethanol (70%) are the additional required solutions.

## 2.3. Isolation of genomic DNA

Genomic DNA isolation using CTAB and slightly changed this method by adding 0.2% PVP to the CTAB extraction buffer to prevent co-isolation of polysaccharides and phenolics. The leaves were frozen in liquid nitrogen and it was carefully ground with clean mortar and pestle. To this extract 1.5 ml (w/v) of pre-heated, (4X CTAB) buffer was added and then incubated at 65 °C for 60 min. The buffer was mixed well and an equal amount of ice-cold chloroform: isoamyl alcohol (24:1) was added. After mixing well, the tubes were centrifuged at 10,000 rpm for 15 min. The volume was measured and 1/10<sup>th</sup> the volume of 3 M sodium acetate and 0.6 ml of ice-cold isopropanol was added and mixed gently to precipitate the nucleic acids. The nucleic acid pellet was picking it up with centrifuged at 10,000 rpm for 15 min and then the supernatant was discarded. The pellet was washed with 70% ethanol, air dried at room temperature. The pellet was dissolved in 50 µl of 1X TE buffer.

## 2.4. DNA purification

DNA solution was treated with 10 µl of RNase was added and then incubated at 37 °C for 1 h, an equal volume of phenol: chloroform: Isoamyl alcohol (25:24:1) was added and mixed well for 5 min. The solution was centrifuged at 10,000 rpm, 4 °C for 15 min. The clear phase was pipetted into a new tube and added 1/10<sup>th</sup> volume of 3 M sodium acetate and DNA precipitated by adding 2.5 ml of ice-cold 99% ethanol. The pellet was air dried then pellet was then re-suspended in 50 µl of 1X TE buffer and stored at -20 °C.

## 2.5. Purity of DNA

The yield of DNA was estimated by using UV-visible spectrophotometer (ELICO-SL-159) at 260 nm. DNA concentrations were calculated by the following formula; Concentration of DNA = O.D at 260 nm \*50\* Dilution. The purity of the DNA was determined by the ratio of absorbance 260 and 280 nm respectively. The quantity of

DNA isolate was checked by running on 0.8% w/v agarose gel with 1 µl of 0.005% ethidium bromide. The obtained bands were matched with lambda DNA marker.

## 2.6. RAPD analysis

RAPD molecular fingerprint using DNA isolated from the different locality of *S. minor*, oligonucleotide from Operon primer was used for PCR amplification to standardize the condition. The PCR reaction mixture was carried out by PCR Thermocycler (Lark Innovation, India). PCR amplification was performed in a total reaction of 20 µl contained 50 mM MgCl<sub>2</sub>, 0.4 µM primer, 2 mM MgCl, 50 ng template DNA, 2 mM of each dNTPs and 1U of Taq Polymerase (HELINI). After a denaturation first step for 3 min at 94 °C, the Second step for each cycle repeated 30 sec at 94 °C, third step for 1 min at 37 °C and a fourth step for 2 min at 72 °C, the amplification mixture was carried out for 39 cycles. The final elongation steps were extended to 10 min. PCR amplification products were run on 1.5% agarose gel in TAE buffer and the gel can also be stained after electrophoresis by soaking in a diluted solution of ethidium bromide. The image of DNA to visualized in the gel documentation system and then photographed under UV light.

## 2.7. Data analysis

RAPD markers for comparing the similarity of *S. minor* on different locality Jaccard's coefficient [14] was used and data scored for their absence '0' or presence '1' of bands for each primer and banding patterns of genotypes for a genotype-specific band, specific primer was identified and poor bands were discarded [15]. The binary data's was employed to assess the polymorphism besides separating the polymorphic bands with the number of scored bands. The polymorphism information contents were estimated by the formula; PIC=2 pi (1-Pi) [16] where Pi is the occurrence of polymorphic bands in various primers. The similarity matrix was constricted by Jaccard's coefficient and the dendrogram was constructed by using the UPGMA (Unweighted pair group method of arithmetic) with help of computer package SPSS ver. 20 [17].

## 3. Results and discussion

DNA isolated from leaves of *S. minor* from 16 different localities and it was extracted by CTAB method, this yielded more and reliable amplification product shown and it's suited for analysis of RAPD by PCR using random decamer primer. Almost all the parameter was tested for RAPD the following, the concentration of DNA, magnesium chloride, primer, dNTPs, Taq polymerase, time intervals for denaturation, annealing, temperature, and elongation of cycles were also optimized for an effect on amplification, and reproducibility of banding pattern is given in (Table 2). The size of the fragment amplified varied from 200 to 2500 bp. The yield of DNA was checked and high qualities have been obtained a range from 1.6 to 1.8 µg/ml. The purity of DNA and quantity amount varies between applications [18]. The primers were screened and obtained polymorphic bands of amplified DNA product for the *S. minor*. The thirteen primers were employed to gained 216 DAN fragment, an average 6 each primer (Table 4). OPB-20 and MAP-02 primers gave the least number (14 bands) of amplified fragment and MAP-05 the most (21 bands). The isolated DNA was compared with random decamer primers the following OPA-11; OPB- OPB-4, OPB-18, OPB-20; OPC-7, OPC-9, OPC-10, OPC-17; OPN-10; OPO-16, MAP-2, MAP-3, and MAP-5 series were used for the analysis of genetic diversity. Among 13 primers were screened and it was produced an average of 5-6 amplicons. Highest amplification was



**Table 2**

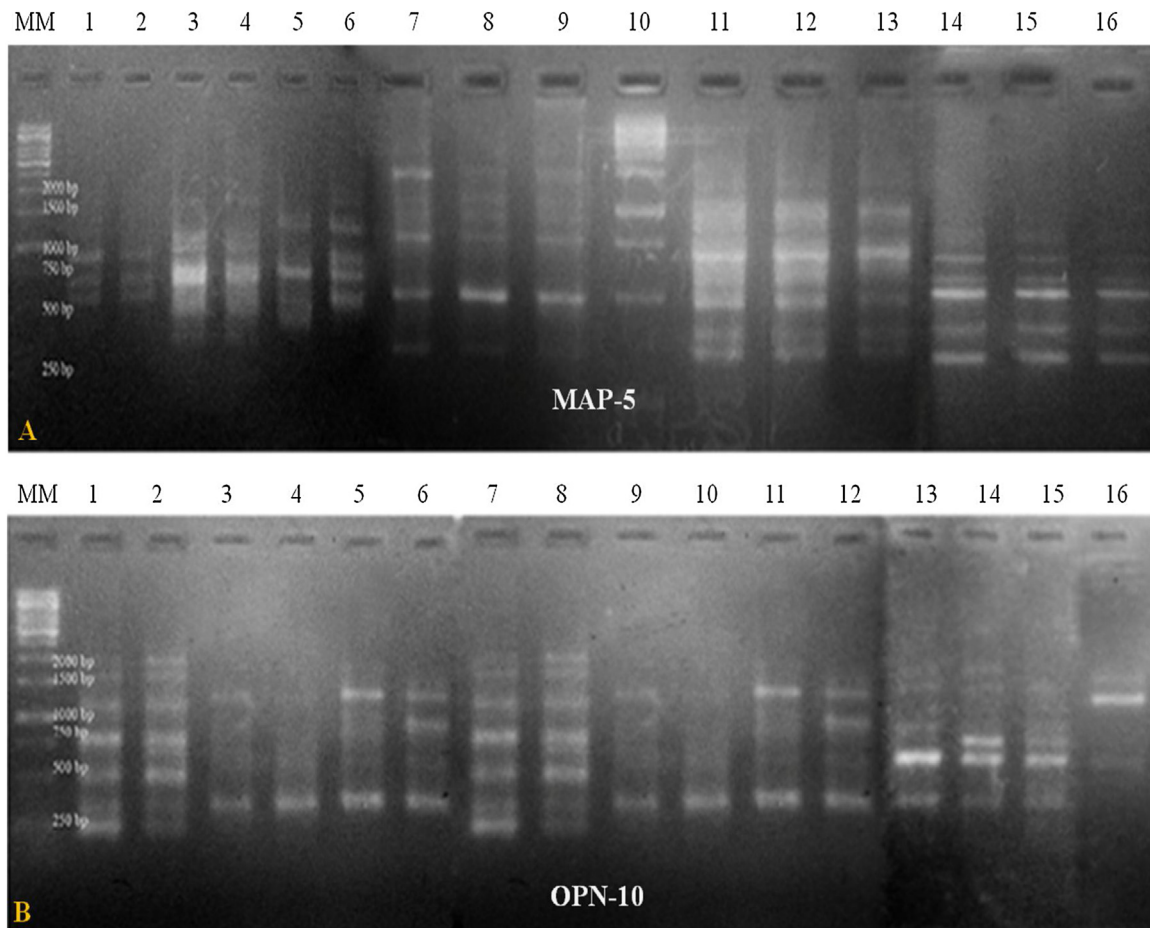
Standardization of PCR conditions were by varying the concentration, variation and the remarks were tabulated.

PCR Parameter	Tested Range	Optimum condition	Remarks
DNA Concentration (ng)	25 ng and 50 ng	50 ng	Absence of amplification with lower concentration and presence of smear at higher concentration affected respectively.
Magnesium Chloride (mM)	2,2.5 and 3	2.5mM	Excess/ Lower concentration increase the non-specificity and yield of the product
Primer concentration ( $\mu$ M)	0.3, 0.4 and 0.5	0.4 $\mu$ M	Lower and higher concentration leads to the absence of amplification and primer-dimer formation respectively.
Taq Polymerase (Units)	0.5 and 1	1U	Lower concentration did not show proper amplification. High concentration showed decreased specificity.
Initial denaturation (min)	2, 3 and 4	94°C for 3.0 min	Lower or Higher time intervals leads to loss of activity in Taq polymerase and lack of reproducibility
Annealing Temperature (°C)	20, 25, 30, 35, 37, 40, 45, 47 and 50	37°C for 30 S	Higher/lower annealing temperature results may differ in specificity
Reaction Volume ( $\mu$ l)	15, 20, 25 and 30	20 $\mu$ l	The cost of the PCR ingredients
Number of Cycles	20, 30, 35, 39, 40, 45 and 50	39	Lower and Higher cycles affect the amplification (from optimum)

obtained in OPB-20, OPC-9, OPC-10, OPN-10, and MAP-5 produced 7-8 bands. The decamer primers were selected for the RAPD analysis, which produced 1086 amplification with an average of 6 amplicons each primer (Table 4).

Highest amplifications were observed in OPA 11 and OPB 4 with 19 amplicons and less number of bands in MAP 2 and OPC 17 of 12 amplicons each. The highest polymorphism in OPN 10 and MAP 5 primers (Fig. 2) showed distinct polymorphism with 13 to 15 amplicons (Table 3) of 65% and 71% respectively. The

overall polymorphisms 74.07% were observed. Monomorphic bands were remarked with OPB-20 with 14 amplicons and overall monomorphism 25.92% (Table 3). The diversity of genetic variables is generally thought of genetic variation among the individual's population of a species or molecular markers [19,20]. RAPD is simplest and the reliable methods used to resolve genetic diversity and similarity [21]. The effectiveness of this marker was reported for the genetic assessment of plant species [22].



**Fig. 2. (A-B)** Banding pattern of *Strychnos minor* of genomic DNA from different localities using thirteen random decamer primers. MM, Molecular marker; 1, Agaram; 2, Oorani; 3, Puthupattu; 4, Thirumanikuzhi; 5, Ramapuram; 6, Thondamannatham; 7, Keelakangeyankuppam; 8, Vagakkolai; 9, Maramadakki; 10, Arasarkulam; 11, Suranviduthi; 12, Manadakulam; 13, Andarkadu; 14, Thettakudi south; 15, Tillaivilagam; 16, Soorakottai.

**Table 3**List of primers, amplified fragments, polymorphic fragment, monomorphic fragment, polymorphism and polymorphism information contents of *S. minor*

Primer	Sequence (5'-3')	Total no. Amplified fragment	No. of Monomorphic fragment	No. of polymorphic fragment	% of polymorphism	Polymorphic information content (PIC)
OPA-11	-CAATCGCCGT-	20	6	14	70	0.29
OPB-04	-GGACTGGAGT-	18	4	14	77	0.40
OPB-18	-CCACAGCAGT-	19	6	13	68	0.38
OPB20	-GGACCCTTAC-	14	4	10	71	0.39
OPN-10	-ACAACGGGG-	20	7	13	65	0.20
MAP-02	-TTGTCTCAGG-	14	4	10	71	0.31
MAP-03	-CGGGATCCGC-	19	6	13	68	0.26
MAP-05	-AAATCGGAGA-	21	6	15	71	0.22
OPC-07	-GTCCCGACGA-	15	4	11	73	0.31
OPC-09	-CTCACCGTCC-	17	3	14	82	0.31
OPC-10	-TGTCTGGGTG-	16	5	11	68	0.27
OPC-17	-TTCCCCCAG-	17	4	13	76	0.22
OPO-16	-TCGGCGGTTT-	16	7	9	56	0.32
<b>Mean</b>						<b>0.29</b>

**Table 4**Summary of Random Amplified Polymorphic DNA amplified product of *S. minor*.

Description	Random Amplified Polymorphic DNA
Total no. of band scored	1086
No. of Monomorphic band	56
No. of Polymorphic band	160
Percentage of Polymorphism	74
No. of primer used	13
Average Polymorphism per primer	8
Average no. of fragment per primers	6
The size range of amplified fragment	200-2500
Total no. of amplified fragment	216

Polymorphism information content (PIC) was estimated from the occurrence of polymorphic bands among all the genotypes were 0.29 (Table 3). The primers OPB 04 showed the higher PIC value of 0.40 followed by the primers OPB 20 (0.39) and OPB 18 (0.38). The primer OPN 10 showed least PIC value of 0.20. The average value of PIC (0.46) was scored RAPD bands for all primers and it's based genetic diversity studies in various crops [23]. Henry, [24] reported to the PIC value provides a measure that is influenced by both the number and frequency of alleles, the maximum PIC value for RAPD marker is 0.40 hence two alleles each locus are assumed in RAPD analysis.

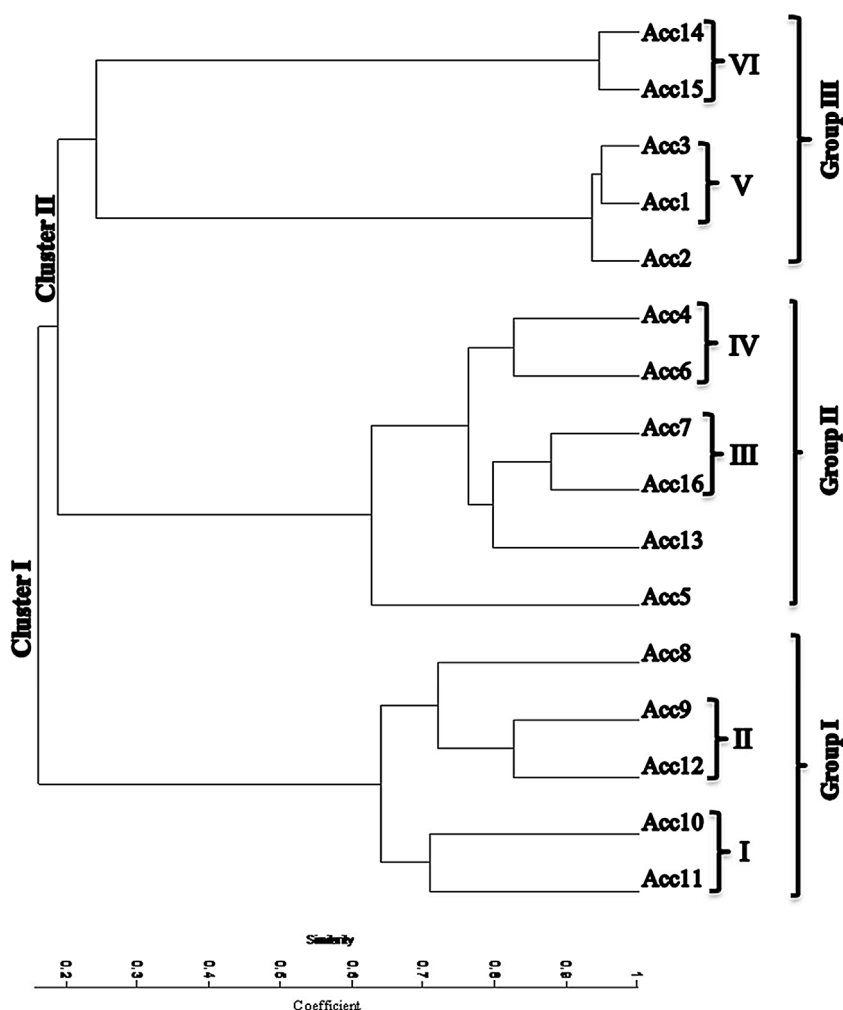
Similarity matrix varied from 0.654 to 0.986 (Table 5). The highest genetic similarities (0.986) were noted between *S. minor* accessions Acc14 and Acc15, whereas the lowest value of (0.654) was obtained

between Acc7 and Acc14 combination. The similarity coefficients based on RAPD markers minimum ranged from 0.654 (between Acc7 and Acc14), 0.666 (between (between Acc5 and Acc14; Acc5 and Acc15) followed by (between Acc1 and Acc7; Acc3 and Acc7; Acc5 and Acc12; Acc7 and Acc15) respectively. Among all Acc7 was different from others, whereas the genetic similarity matrix was maximum among sixteen accessions ranging from 0.986 (between Acc14 and Acc15) followed by 0.984 (Acc1 and Acc2). The relatedness among 5 accessions (Acc2, Acc7, Acc12, Acc14, and Acc15) within sixteen accessions was found out and genetic similarity among sixteen accessions (Table 5). The previous study reported that a similar range in similarity coefficient values had also been measured in plant species [25]. Cluster analysis (UPGMA) of the similarity coefficient built a dendrogram (Fig. 3) which showed the overall genetic closeness among the genotypes. The RAPD has simplified and its use in the analysis of genetic diversity in several plant genera [26]. [27] reported the number of migrants and higher values among the populations were calculated has been described.

The genetic variability between sixteen localities was shown by the UPGMA dendrogram (Fig. 3). The sixteen accessions were segregated into clusters consisting of two or major groups of two, cluster (I) and cluster (II) respectively by RAPD markers. Among accessions cluster (I) sub-divided into two, Acc10 and Acc 11 followed by Acc9 and Acc12 in Group (I). Cluster (II) divided two major groups, Group (II) and Group (III). Group (II) further subdivided into two, Acc4 and Acc6 and then Acc7 and Acc16 in the second group. The third group also divided two, one was Acc1 and Acc3 followed by Acc14 and Acc15 in (Group III), while other Accessions belong to different groups (Acc2, Acc5, Acc8, and Acc13)

**Table 5**Jaccard's similarity matrix of *S. minor* from different locality using RAPD markers

	Acc1	Acc2	Acc3	Acc4	Acc5	Acc6	Acc7	Acc8	Acc9	Acc10	Acc11	Acc12	Acc13	Acc14	Acc15	Acc16
<b>Acc1</b>	1.000															
<b>Acc2</b>	0.984	1.000														
<b>Acc3</b>	0.981	0.979	1.000													
<b>Acc4</b>	0.723	0.726	0.723	1.000												
<b>Acc5</b>	0.704	0.708	0.697	0.861	1.000											
<b>Acc6</b>	0.719	0.723	0.719	0.960	0.881	1.000										
<b>Acc7</b>	0.670	0.674	0.670	0.943	0.910	0.951	1.000									
<b>Acc8</b>	0.682	0.678	0.689	0.726	0.6783	0.751	0.719	1.000								
<b>Acc9</b>	0.678	0.674	0.686	0.686	0.674	0.712	0.686	0.946	1.000							
<b>Acc10</b>	0.674	0.678	0.682	0.740	0.701	0.765	0.733	0.933	0.905	1.000						
<b>Acc11</b>	0.674	0.678	0.682	0.740	0.701	0.765	0.726	0.928	0.936	0.953	1.000					
<b>Acc12</b>	0.682	0.678	0.689	0.689	0.670	0.715	0.682	0.938	0.956	0.918	0.938	1.000				
<b>Acc13</b>	0.708	0.712	0.708	0.933	0.921	0.960	0.968	0.733	0.701	0.747	0.74	0.697	1.000			
<b>Acc14</b>	0.708	0.719	0.715	0.686	0.666	0.697	0.654	0.704	0.701	0.712	0.697	0.697	0.678	1.000		
<b>Acc15</b>	0.715	0.726	0.723	0.693	0.666	0.704	0.670	0.712	0.715	0.719	0.704	0.712	0.678	0.986	1.000	
<b>Acc16</b>	0.682	0.686	0.682	0.941	0.870	0.938	0.960	0.758	0.719	0.771	0.758	0.715	0.946	0.674	0.689	1.000



**Fig. 3.** Hierarchical clustering analysis of *Strychnos minor* based on Jaccard's similarity matrix obtained by UPGMA.

Acc1, Agaram; Acc2, Oorani; Acc3, Puthupattu; Acc4, Thirumanikuzhi; Acc5, Ramapuram; Acc6, Thondamannatham; Acc7, Keelakangeyankuppam; Acc8, Vagakollai; Acc9, Maramadakki; Acc10, Arasarkulam; Acc11, Suranviduthi; Acc12, Manadakulam; Acc13, Andarkadu; Acc14, Thettakudi south; Acc15, Tillaivilagam; Acc16, Soorakottai

respectively. Therefore, the marker RAPD can be effectively used in the determination of genetic associations with *S. minor* population. However, [28] assumed that RAPD is possible a better tool for phylogenetic study.

#### 4. Conclusion

The RAPD method successfully discriminates have bought valuable to the taxonomy of the plants species. Molecular characterization by RAPD markers could be helpful for rapid identification of plant's species and also comparing with different species. Therefore, our present findings provide guidance for proper identification of these plant species growing an extremely arid environment, to help in their subsequent and sustainable utilization for combat human and natural pressure on these endangered natural resources.

#### Conflicts of interest

Authors have declared no conflicts of interest

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