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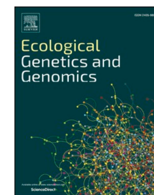

Genetic relationship and polymorphism of selected medicinal plants of Asclepiadaceae using RAPD molecular analysis method

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A R T I C L E I N F O

Keywords:
Asclepidaceae
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Oxystelma esculentum
Pentatropis capensis

A B S T R A C T

Present study was aimed to assess the genetic similarity among the medicinal plants of Asclepiadaceae family members such as *Ceropegia juncea, Gymnema sylvestre, Oxystelma esculentum, Pentatropis capensis* and *Wattakaka volubilis*, by using RAPD based molecular analysis. Sixteen primers (OPA, OPC, OPF and OPG series) were used to screen the genetic variation between the selected Ascleipidaceae members. RAPD analysis revealed the presence of 936 bands in which 466 were monomorphic and 256 were polymorphic bands. In particular, 49.78% were monomorphic and 27.35% were polymorphic with an average of 16% polymorphism per primer from the 936 amplified products. The highest number of bands was observed in *Ceropegia juncea* (198) followed by *Pentatropis capensis* (190), *Oxystelma esculentum* (189), *Gymnema sylvestre* (182) and *Wattakaka volubilis* (177). Among the primers used OPC 05 (17 bands), OPC 16 (17 bands) and OPC 03 (18 bands) produced maximum number of bands in *Wattakaka volubilis*, *Pentatropis capensis* and *Gymnema sylvestre* respectively. UPGMA cluster analysis revealed that presence of *Wattakaka volubilis* and *Gymnema sylvestre* in a same cluster (cluster 1) and *Pentatropis capensis* and *Oxystelma esculentum* in same cluster (cluster 2) had close relationship with each other. Findings revealed that presence of *Ceropegia juncea* in separate sub cluster (2) clearly evidences that species is so distant from other species of Asclepiadaceae. It may be concluded that RAPD tool will be very much helpful for the taxonomist to identify the plant members and place them in the appropriate family through molecular data.

1. Introduction

In molecular biology field, modern tools play a vital role in analyzing genetic diversity, population genetics and genetically characterization in various plant species and cultivars. Due to domestication process, genetic variation in crop plants decreased due to continuous selection pressure for traits such as great yield or disease resistance. It plays a crucial role in both management and conservation programs. But it is necessary to compare the genetic composition of the germplasm of existing cultivars to their ancestors and related species [1]. Medicinally important plants serve as hub for isolating biologically active compounds which possess multiple mode of action. At present, there is a serious threat of extinction and severe genetic loss of medicinal plants due to utilization for drug preparation. Medicinal plants such as *Bacopa monnieri* [2], *Aloe* sp. [3], *Strychnos minor* [4], *Prunus africana* [5], *Rauwolfia tetraphylla,* [6], *Chlorophytum* sp. [7], *Inula royleana* [8,] *Hemigraphis colorata, Marjorana hortensis, Artemisia vulgaris, Artemisia pallens, Ocimum sanctum, Ocimum basilicum, Ocimum hratissimum, Mentha piparita, Mentha citrate, Mentha spicata, Acorus calamus, Centella asiatica, Bacopa moninierii, Piper longum, Piper nigrum, Clitoria ternatea, Aloe vera,*

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Stevia rebaudia [9] etc., which possess therapeutical values for number of diseases must be conserved through molecular techniques for gene bank collection. For most of these endangered medicinal plant species, effective conservation plans are minimal and little material is available in gene banks. Morphological inspection is quite simple and direct way to study diversity, from which we cannot get significant genetic diversity among accessions [10]. For improvement of the breeding and cultivation of plants, understanding its genetic assortment is especially important. In recent studies, the use of molecular markers, revealing polymorphism at the DNA level, has been playing an increasing part in plant biotechnology and their genetic studies. DNA markers seem to be the best candidates for efficient evaluation and selection of plant material. Unlike protein markers, DNA markers segregate them as single genes and they are not affected by the environment. These DNA based markers are differentiated in two types first non PCR based (RFLP) and second is PCR based markers (RAPD, AFLP, SSR, SNP etc.). Random amplified polymorphic DNA (RAPD) marker system has been extensively used to distinguish the genetic variation at molecular level in several medicinal and aromatic plants [11]. Similarly, the studies on molecular analysis demonstrated that RAPD markers are particularly useful tools to compare the genetic relationship and pattern of variation among several prioritized and endangered medicinal plants [12]. RAPD based genetic analysis has been made in between species level in *Tinospora* [13,14], *Ceropegia* [15–18]; *Gymnema* [19,20], *Pentatropis* [21], *Wattakaka* [22,23]. Based on findings observed from our previous ethnobotanical studies, Asclepiadaceae were the most represented family used for different ailments by the rural people of Madurai district, Tamil Nadu, India [24]. In addition, as per our knowledge and literature survey, there is a lack of study on the molecular characterization of genetic similarity of Asclepiadaceae family. The present study was aimed to assess the genetic similarity among the medicinal plants viz., *Ceropegia juncea, Gymnema sylvestre, Oxystelma esculentum, Pentatropis capensis* and *Wattakaka volubilis* by using RAPD based molecular analysis.

2. Materials and methods

2.1. Studies on molecular characterization (RAPD analysis)

2.1.1. Collection of plant materials

Collected plant species *Ceropegia juncea, Gymnema sylvestre*, *Pentatropis capensis*, *Oxystelma esculentum* and *Wattakaka volubilis* were identified and authenticated by a botanist working in Botanical Survey of India, Coimbatore, India. Three samples of young plant materials (shoot tips of *Ceropegia juncea* and leaves of *Gymnema sylvestre*, *Pentatropis capensis*, *Oxystelma esculentum* and *Wattakaka volubilis*) of selected plants were collected early morning from the Botanical garden (maintaining wild species collected from natural habitats) of Saraswathi Narayanan College (Autonomous), Madurai, Tamil Nadu, India. These were kept between moist tissue paper in a plastic bag and kept away from sunlight. The leaves were de-starched by covering them for 24–48 h before use. Leaves were then frozen immediately in liquid nitrogen until required.

2.1.2. Reagents and chemicals used

The chemicals and reagents used in the isolation of DNA were: CTAB extraction buffer [2% (w/v) CTAB; 10 mM EDTA, pH 8.0; 100 mM Tris–HCl, pH 8.0; 1.5 M NaCl]; CTAB/NaCl solution [10% (w/v) CTAB; 0.7 M NaCl mixed at 65 °C with stirring]; CTAB precipitation solution [1% (w/v) CTAB; 50 mM Tris–HCl, pH 8.0; 10 mM EDTA]; high salt TE buffer [10 mM Tris–HCl, pH 8.0; 0.1 mM EDTA, pH 8.0; 1.0 M NaCl]; TE buffer [1 M Tris–HCl, pH 8.0; 0.5 M EDTA, pH 8.0]; Phenol: Chloroform: Isoamyl alcohol (25:24:1); chloroform: isoamyl alcohol (24:1, v/v); ice cold iso-propanol; 80% ethanol; polyvinylpolypyrrolidone (PVPP); 2-mercaptoethanol (2 ME) and liquid nitrogen.

2.1.3. DNA isolation

Total genomic DNA was extracted using a modified CTAB method based on the method described by Doyle and Doyle (1990) & Hills and Staden (2002) [25,26]. One gram of freshly harvested plant samples were taken, washed under running tap water and then dried on filter paper. These plant samples were ground in liquid nitrogen using a mortar and pestle along with 50 mg of PVP and were made to fine powder. The powder was quickly transferred to centrifuge tubes, added 5 ml of freshly prepared preheated (65 °C) DNA extraction buffer to each tube and shaken vigorously by inversion to form slurry. The tubes with samples were incubated at 65 °C in water bath for 1 h with intermittent shaking and allowed to cool at room temperature. An equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and mixed properly by inversion and centrifuged at 12000 rpm for 15 min.at 4 °C. The supernatant was transferred to fresh 2 ml microfuge tube. An equal volume of Chloroform: Isoamyl alcohol (24:1) was added and centrifuged at 11000 rpm for 10 min to separate the phases. The supernatant was carefully transferred to a new 2 ml tube and added equal volume of 3 M sodium acetate and ice cold isopropanol. Then the mixture was gently mixed and incubated at −20 °C to overnight. The incubated samples were centrifuged at 12000 rpm for 15 min and then supernatant was discarded. A pellet of white fibrous structure of DNA was observed in tube. The pellet was washed with 100 μl of 70% ethanol to remove the impurities, and then centrifuged at 5000 rpm for 5 min. The supernatant was discarded, and the pellet was air dried. After drying, 100 μl of TE buffer was added with the pellets. Then the pellet was dissolved in TE buffer and treated with 3 μl RNase. The DNA was maintained at −20 °C for short term storage for further use.

2.1.4. Quantity and purity of DNA

The yield of DNA per gram of plant tissue extracted was measured using a UV spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the samples on 1.8% agarose gel and based on the intensities of band when compared with the Lambda DNA marker (Fermentas, USA).

2.1.5. Screening of primers

Sixty primers of different RAPD series (Operon Tech. Alameda, USA) were used to analyze the genetic variation among the sample plants. These primers were from OPA, OPC, OPF and OPG series. Only the random primers of OPC series gave amplification profiles. Twenty random primers of OPC series (OPC 01 to OPC 20) were tested for RAPD analysis. Scoreable amplification profiles were given by all above mentioned primers of OPC01, OPC02, OPC03, OPC05, OPC06, OPC07, OPC08, OPC11, OPC12, OPC13, OPC14, OPC15, OPC16, OPC17, OPC18 and OPC20. The sequence of sixteen primers used in this study are given

Table 1
Details of sequence of sixteen primers (OPC series) used in RAPD analysis.

Sl. No	Primers	Sequences (5’ – 3′)
1	OPC-01	TTCGAGCCAG
2	OPC-02	GTGAGGCGTC
3	OPC-03	GGGGGTCTTT
4	OPC-05	GATGACCGCC
5	OPC-06	GAACGGACTC
6	OPC-07	GTCCCGACGA
7	OPC-08	TGGACCGGTG
8	OPC-11	AAAGCTGCGG
9	OPC-12	TGTCATCCCC
10	OPC-13	AAGCCTCGTC
11	OPC-14	TGCGTGCTTG
12	OPC-15	GACGGATCAG
13	OPC-16	CACACTCCAG
14	OPC-17	TTCCCCCCAG
15	OPC-18	TGAGTGGGTG
16	OPC-20	ACTTCGCCAC

in Table 1.

2.1.6. 6: RAPD analysis

RAPD analysis was carried out using 16 standard decamer oligonucleotide primers viz., OPC01, OPC02, OPC03, OPC05, OPC06, OPC07, OPC08, OPC11, OPC12, OPC13, OPC14, OPC15, OPC16, OPC17, OPC18 and OPC 20 (Operon Tech. Alameda, USA). The RAPD analysis was performed as per the standard method of Williams (1990) [27]. Each amplification reaction mixture of 25 μl contained 2.5 μl (20 ng) of template DNA, 3.0 μl of 10X assay buffer (100 mM Tris- HCl, pH 8.3, 0.5 M KCl, 1.5 mm $MgCl_2$ and 0.01% gelatin), 2.5 μl of each of dNTPs, 2.5 μl of primer and 1.0 μl of TaqDNA polymerase and 13.5 μl of nucleus free water (NFW), (Bangalore Genei Pvt. Ltd., Bangalore, India). The amplification was carried out in a thermal cycler (Biorad, USA). The first cycle consisted of initial denaturation of template DNA at 94 °C for 5 min, denaturation of template DNA at 94 °C for 45 s, primer annealing at 40 °C for 45 s and primer extension at 72 °C for 45 s. In the subsequent 44 cycles, the period of denaturation was reduced to 1 min while the primer annealing and primer extension time were maintained same as in the first cycle. The final cycle consisted of only primer extension at 72 °C for 8 min. The PCR products were separated on a 1.8% agarose gel containing ethidium bromide (0.5 μg/ml of gel solution). The size of the amplicons was determined using size standards (100 bp DNA ladder plus or DNA ladder mix, MBI Fermentas, Graiciuno, Vilnius, Lithuania). DNA fragments were visualized under UV transilluminator (Syngne, USA) and the gels were photographed using UV gel documentation system.

2.1.7. Data scoring and analysis

Evaluation of fragment patterns was carried out by similarity index. Reproducible bands were scored manually as ‘1’ or ‘0’ for the presence or absence of the bands. Polymorphic information content (PIC) values were calculated for each RAPD primer according to the formula: $PIC = 1 - \sum (P_{ij})^2$, where P_{ij} is the frequency of the i th pattern revealed by the jth primer summed across all patterns revealed by the primers [28]. The final RAPD data generated were used to calculate pairwise similarity co-efficient [29] using the similarity for qualitative data (SIMQUAL) format of NTSYS-pc version 2.1 (numerical taxonomy and multivariate analysis system) software package [30]. Cluster analysis was performed on the basis of genetic similarity matrix, and the resulting similarity co-efficient were used for constructing dendrogram using the unweighted pair group method with arithmetic average (UPGMA) with the SAHN module of NTSYS-pc [31]. The similarities between matrices based on different marker systems (RAPD) were calculated using the standardized Mantel co-efficient [32].

3. Results

In the present study, genomic DNA was isolated from plant materials (leaves and shoot tips) of *Wattakaka volubilis, Gymnema sylvestre*, *Pentatropis capensis, Oxystelma esculentum* and *Ceropegia juncea* obtained from their growing regions of Madurai, TamilNadu, India. The isolated genomic DNA of respective plants was run on 1.8% agarose gel after treatment with RNase A for determining the quality and quantity of DNA. In RAPD analysis, high quality of DNA is required for PCR based amplification purpose; therefore, good quality of DNA was confirmed by agarose gel electrophoresis. By using this genomic DNA as a template, amplification was carried out by using different RAPD primers of OPC series. PCR amplification was carried out by using sixteen random RAPD primers (OPC01, OPC02, OPC03, OPC05, OPC06, OPC07, OPC08, OPC11, OPC12, OPC13, OPC14, OPC15, OPC16, OPC17, OPC18 and OPC20) and they were found reproducible and satisfactory. The other primers used exhibited smear and unreadable band pattern and therefore not taken for analysis. Amplified products were confirmed by running PCR products on 1.8% agarose in 0.5X TAE at constant voltage. The size range of all the amplified fragments produced by the aforementioned primers was 100–1000 bp.

In total, 936 bands were produced by all the primers used in this study. Among these 936 bands, 466 monomorphic and 256 polymorphic bands were observed (Figs. 1 and 2; Table 2). Therefore, out of 936 amplified products, 49.78% were monomorphic and 27.35% were polymorphic with an average of 16% polymorphism per primer. The highest number of bands was produced by OPC 03 (74 bands) followed by OPC 05 (71 bands) and lowest number of bands was produced by OPC 14 (40 bands), OPC 06 (41bands) and OPC 20 (44 bands). The average number of bands per primer was 58. Maximum polymorphism was observed in OPC 16 (39.72%) and followed by OPC 17 (38.71%) and OPC 18 (36.36%) and minimum polymorphism was observed in OPC 11 (18.18%) followed by OPC 06 (19.51%) and OPC 02 (20.00%). Moreover, the RAPD primers used in this study also showed rare (93 bands) and unique (121 bands) fragments of different molecular sizes. The number of such bands was also varied with respect to different primers used and the species selected for the study. The primer OPC 13 showed maximum of 15 rare bands and only 9 unique bands. All other primers showed 5–8 rare bands except OPC 06. The primers OPC 01 to OPC 05 showed 10–15 unique bands and the other primers had number of such bands between 5 and 9. The unique bands were found to be high while using OPC 03 (15) and followed by OPC 01 (13) and OPC 05 (12). The number of bands while using 16 primers for each plant species was varied from 177 to 198. The highest number of bands was observed by *Ceropegia juncea* (198 bands) followed by *Pentatropis capensis* (190 bands), *Oxystelma esculentum* (189 bands) and *Gymnema sylvestre* (182 bands). The lowest number of bands was scored by *Wattakaka volubilis* (177 bands). Among the primers used OPC 05 (17 bands), OPC 16 (17 bands) and OPC 03 (18 bands) produced maximum number of bands in *Wattakaka volubilis*, *Pentatropis capensis* and *Gymnema sylvestre* respectively. In the species, *Oxystelma esculentum* (17 bands) and *Ceropegia juncea* (16 bands) produced high number of bands while using the primer OPC 01 (Tables 3 & 4).

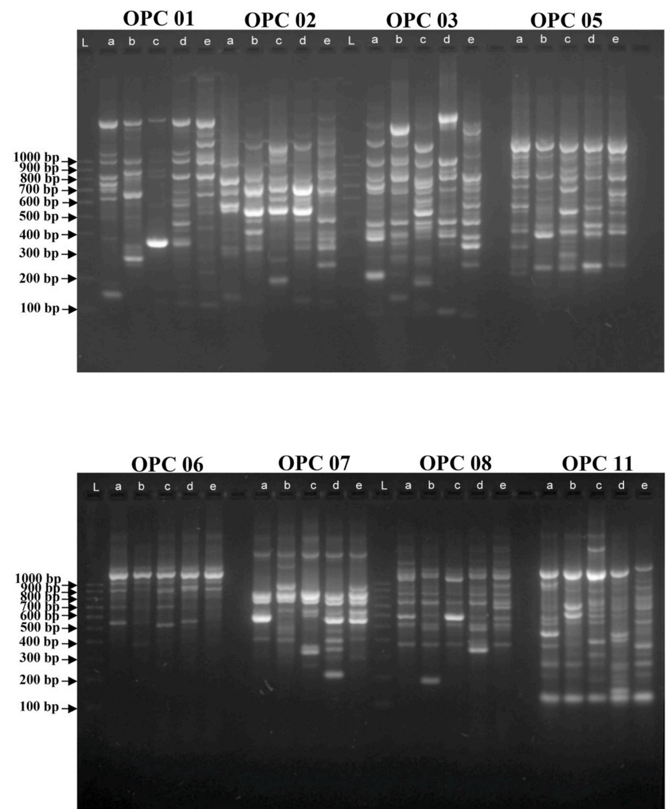

Fig. 1. RAPD amplification profiles of five different species of Asclepiadaceae with different OPC primers.

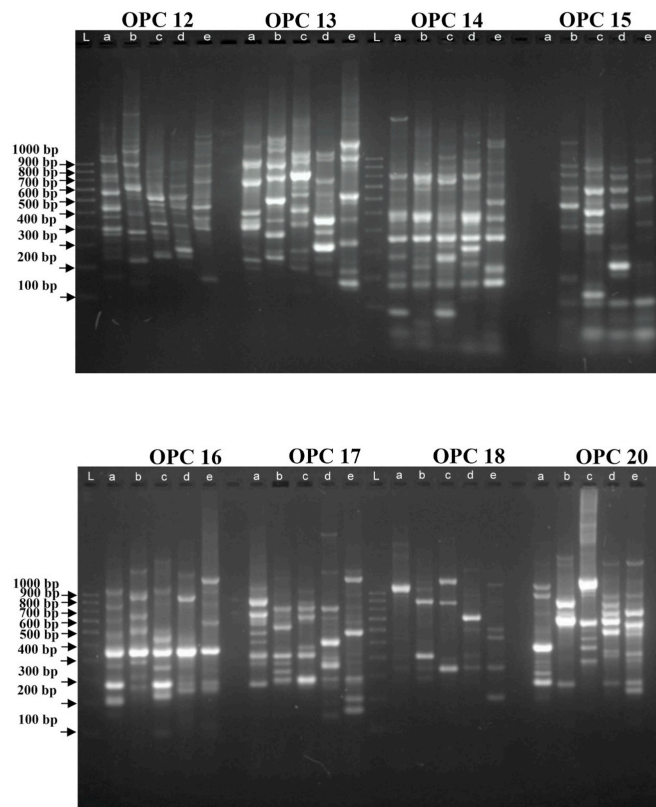

Fig. 2. RAPD amplification profiles of five different species of Asclepiadaceae with different OPC primers.

3.1. b. Cluster analysis of bands produced by all primers

The genetic similarity indices were developed on the basis of amplified products of sixteen RAPD primers with samples of five different plant species (*Wattakaka volubilis, Gymnema sylvestre*, *Pentatropis capensis, Oxystelma esculentum* and *Ceropegia juncea*). The genetic similarity coefficients are shown in Table 3. The genetic similarity coefficient values were ranging from 0.45 to 0.64 with the mean of 0.55. The lowest similarity value was present between the sample number 3 (*Gymnema sylvestre*) and 5 (*Ceropegia juncea*), while the highest similarity value was found between sample number 2 (*Pentatropis capensis*) and 4 (*Oxystelma esculentum*). UPGMA cluster analysis has also revealed the same results. In cluster analysis of all primers sample number, 2 and 4 were highly similar while sample number 3 (*Gymnema sylvestre*) and 5 (*Ceropegia juncea*) had shown very low similarity level. The results revealed that both of them (*Gymnema sylvestre* and *Ceropegia juncea*) were so distant from each other as they were present in two different clusters.

UPGMA cluster analysis of all primers revealed two major clusters in this cladogram (Fig. 3). Data from sixteen primers of OPC series were analyzed in this cluster analysis and samples had showed 63% similarity level and 37% divergence among them. Cluster 1 was characterized by samples of *Wattakaka volubilis* and *Gymnema sylvestre* (1 and 3). The samples of *Wattakaka volubilis* (1) were clustered together in cluster 1. All samples of *Gynema sylvestre* (3) were also clustered together in cluster 1. 52% similarity level was present in all the samples of cluster 1. Cluster 1 was further divided into sub cluster 1 and sub cluster 2. Sub cluster 1 contained all samples of *Wattakaka volubilis* and they had shown 56% similarity. The sub cluster 2 contained all samples of *Gymnema sylvestre* which showed 56% similarity. It is obvious that, both of them had shown 56% similarity. The inclusion of samples of *Wattakaka volubilis* and *Gymnema sylvestre* exhibited close relationship between them and or noticed them as least diverse species of Asclepiadaceae.

In cluster 2, samples of *Pentatropis capensis, Oxystelma esculentum and*

Table 3
Total number of bands produced from 16 primers of OPC series during RAPD analysis of selected species of Asclepiadaceae.

S. No	Primers	W	P	G	O	C	Total bands
1	OPC 1	13	15	6	17	16	67
2	OPC 2	12	9	11	13	15	60
3	OPC 3	14	16	18	13	13	74
4	OPC 5	17	13	14	13	14	71
5	OPC 6	9	8	8	9	7	41
6	OPC 7	9	14	10	12	11	56
7	OPC 8	13	15	10	15	15	68
8	OPC 11	13	11	13	14	15	66
9	OPC 12	9	9	12	7	9	46
10	OPC 13	10	11	7	13	13	54
11	OPC 14	8	10	7	8	7	40
12	OPC 15	12	8	13	9	11	53
13	OPC 16	14	17	11	12	14	68
14	OPC 17	12	11	13	11	15	62
15	OPC 18	12	12	17	12	13	66
16	OPC 20	0	11	12	11	10	44
	Total	177	190	182	189	198	936

W - *Wattakaka volubilis*, P - *Pentatropis capensis*, G - *Gymnema sylvestre*, O - *Oxystelma esculentum* and C - *Ceropegia juncea.*

Table 2
Details of number of bands generated and percentage of polymorphism as revealed by RAPD primers used against species of Asclepiadaceae.

S. No	Primers	Oligo sequence	Total Bands	Monomorphic bands	Polymorphic bands	Rare bands	Unique bands	Polymorphism %
1	OPC 1	5′TTCGAGCCAG3′	67	26	22	6	13	32.84
2	OPC 2	5′GTGAGGCGTC3′	60	33	12	5	10	20.00
3	OPC 3	5′GGGGGTCTTT3′	74	31	20	8	15	27.03
4	OPC 5	5′CCGCATCTAC3′	71	38	15	6	12	21.14
5	OPC 6	5′GATGACCGCC3′	41	28	8	0	5	19.51
6	OPC 7	5′GAACGGACTC3′	56	28	14	5	9	25.00
7	OPC 8	5′GTCCCGACGA3′	68	42	17	3	6	25.00
8	OPC 11	5′TGGACCGGTG3′	66	39	12	7	8	18.18
9	OPC 12	5′CTCACCGTCC3′	46	29	10	3	4	21.74
10	OPC 13	5′TGTCTGGGTG3′	54	17	13	15	9	24.08
11	OPC 14	5′AAAGCTGCGG3′	40	22	10	4	4	25.00
12	OPC 15	5′TGTCATCCCC3′	53	24	18	6	5	33.96
13	OPC 16	5′AAGCCTCGTC3′	68	30	27	6	5	39.72
14	OPC 17	5′TGCGTGCTTG3′	62	28	24	6	4	38.71
15	OPC 18	5′GACGGATCAG3′	66	28	24	8	6	36.36
16	OPC 20	5′CACACTCCAG3′	44	23	10	5	6	27.74
	All Primers	Total	**936**	**466**	**256**	**93**	**121**	**27.35**

Table 4
Genetic similarity coefficients of five species of Asclepiadaceae.

	Wattakaka sp.	*Pentatropis* sp.	*Gymnema* sp.	*Oxystelma* sp.	*Ceropegia* sp.
Wattakaka sp.	1.0000000				
Pentatropis sp.	0.5803922	1.0000000			
Gymnema sp.	0.5647059	0.5294118	1.0000000		
Oxystelma sp.	0.5294118	0.6352941	0.5490196	1.0000000	
Ceropegia sp.	0.4901961	0.6117647	0.4470588	0.6156863	1.0000000

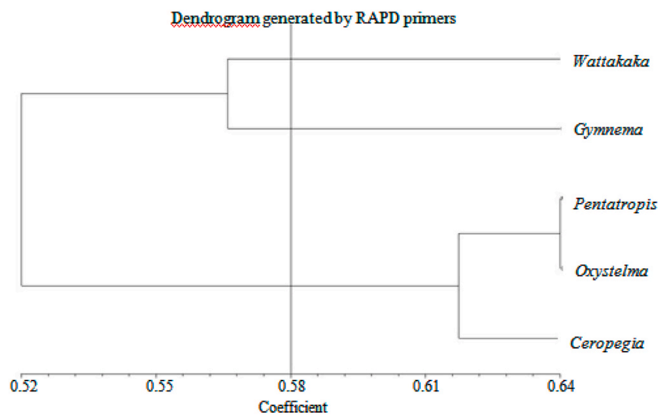

Fig. 3. Dendrogram produced by amplified products of sixteen primers of OPC series used for RAPD analysis of selected medicinal species of Asclepiadaceae.

Ceropegia juncea were clustered. They had shown 62% similarity with each other. However, cluster 2 was further divided into sub cluster 1 and sub cluster 2. The sub cluster 1 contained samples of *Pentatropis capensis and Oxystelma esculentum*. These two groups stand with similarly of 62%. They were the least diverse samples because they were at 62% similarity level. Cluster 2 was also characterized by sub cluster 2 by samples of *Ceropegia juncea* (5). They had shown 62% similarity within the samples of cluster 2. The sub cluster 2 was formed in cluster 2, reveals distant relationship with the other species in this study. Among the five species, *Ceropegia juncea* (sample 5) was the most diverse plant species of *Asclepiadaceae.* UPGMA cluster analysis had shown a clear picture about the position of five genera. Presence of *Wattakaka volubilis* and *Gymnema sylvestre* in a same cluster (cluster 1) had revealed their close relationship with each other. Similarly, presence of *Pentatropis capensis* and *Oxystelma esculentum* in same cluster (cluster 2) showed very close relationship of them. However, the presence of *Ceropegia juncea* in separate sub cluster [2] in cluster 2 evidences that this species is so distant from other species of *Asclepiadaceae* (*Wattakaka volubilis, Gymnema sylvestre*, *Pentatropis capensis* and *Oxystelma esculentum*) selected in this study.

4. Discussion

In our study, among the 936 bands produced, 466 were monomorphic and 256 were polymorphic bands. Among the primers used OPC 05 (17 bands), OPC 16 (17 bands) and OPC 03 (18 bands) produced maximum number of bands in *Wattakaka volubilis*, *Pentatropis capensis* and *Gymnema sylvestre* respectively. But, *Oxystelma esculentum* (17 bands) and *Ceropegia juncea* (16 bands) produced high number of bands while using the primer OPC 01. Similarly, in another study, maximum number of bands was produced by OPC 04 and minimum number of bands was produced by OPC 08 (15 bands). The variation in the number of bands amplified by different primers might be influenced by variable factors such as primer structure, template quantity and less number of annealing sites in the genome [33]. Moreover, the unique bands (10-15) formed by amplification through OPC primers (OPC 01 to OPC 05) in our molecular analysis could be more informative in classification [34].

In recent years, molecular fingerprinting has been widely practiced to scrutinize the genetic diversity and genetic characterization between plant species, crop cultivars and sub family in a particular family. Molecular fingerprinting also plays a vital role in detecting the genetic similarity between different plant genus levels within the same family itself. 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]. The diversity of genetic variables is generally thought of genetic variation among the individual's population of a species or molecular markers [35]. RAPD is simplest and the reliable methods used to resolve genetic diversity and similarity [36]. In our study, effectiveness of the marker was reported for the genetic assessment of family species. These methods are favored because they are independent of the developmental stage of the plant [37]. Molecular investigation of non-coding cpDNA markers (trnT-L and trnL-F spacers), and the trnL intron in asclepiadoideae recorded that two Asclepiadoideae tribes, Ceropegiae and Marsdenieae possessed erect pollina [38]. Similarly, several authors have also analyzed genetic variations among different plant species belonging to Apocynaceae [38]; Rutaceae [39]; Malvaceae [40]; Lemnaceae [41]; Araliaceae [42].

Level of polymorphism within the species and members of different species of Apocyanaceae were evaluated by Tariq et al. (2011)(22) and found that total 105 monomorphic and 272 polymorphic bands were produced from all primers and out of 322 amplified products, 26% were monomorphic and 68% were polymorphic. Genetic fingerprinting of 11 plant species of desert origin (*Andrachne telephioides, Zilla spinosa, Caylusea hexagyna, Achillea fragrantissima, Lycium shawii, Moricandia sinaica, Rumex vesicarius, Bassia eriophora, Zygophyllum propinquum* subsp *migahidii, Withania somnifera, and Sonchus oleraceus*) exhibited a total of 164 bands with well-defined and major bands for a single plant species for a single primer ranged from 1 to 10. All the bands clearly discriminated between each plant species for easy identification, conservation and sustainable use of these plants (Arif et al. (2010) [43]. The genetic similarities were derived from the dendrogram constructed by the pooled RAPD data exhibited clear grouping of species under their respective genera, inter- and intra-generic classification between 9 constituent species belonging to 5 traditionally recognized genera under the tribe Millettieae and Leguminosae [44]. Degree of polymorphism occurs during RAPD analysis can be due to genetic drift due to isolations of populations and limiting the intra specific diversity. Other influential factors such as fragmentation disorder the natural or native habitat which subsequently decreases in gene flow between populations [45]. It can also be inferred that amount of pollen, its size, genetic composition of pollen, pollinator behavior, mating system, in breeding and out breeding play a vital role in influencing the gene flow resulting in polymorphism. Moreover, studies of marker genes in the progeny generation may lead to a confusion due to pollen transfer with both post-pollination pre zygotic events (e.g. self-incompatibility responses) and post-zygotic events (e.g. embryo abortion, inbreeding depression and seedling mortality) [46]. Occurrence of qualitative mutation leads to sudden changes in morphological, anatomical and biochemical features whereas quantitative mutation express smaller and gradual changes in plant genome and resulting in altered phenotype [47]. In this study, *Ceropegia* sp. could have been exposed to various environmental factors such as outbreeding, pollen genetic composition, zygotic events,

occurrence of mutations etc., resulting in polymorphism (falling on separate cluster). But, the high genetic variation of 60% found between individuals within the natural populations and 40% of variations found among populations clearly indicates that there has not been much genetic mixing between different them. Therefore, the gene pool is not likely to be threatened immediately.

All five species which were assessed in this present study share same habitat and occur sympatrically. *Gymnema sylvestre, Oxystelma esculentum* and *Pentatropis capensis* are from tribe Asclepiadeae but *Wattakaka volubilis* from tribe Marsdenieae. This could have affected the evolutionary processes to produce the resulting like-patterns of the overall variability within the constituent species and taxa. The variations might have occurred at interspecific levels by the process of pollination with same insects and due to same soil and climatic conditions. Therefore, the observations suggest that all the studied species are the members of subfamily of Asclepiadoideae with 56–63% similarity among them with low genetic diversification. The main reason for low genetic diversification may be due to evolutionary process by adapting to the most suitable environmental conditions. However, the other species *Ceropegia juncea* is placed in tribe Ceropegeae. Studies had revealed that tribes Marsdenieae and tribe Asclepiadeae are the most prominently *Diptera* serviced groups [48]. In previous investigations, by using RAPD analysis considerable genetic variation (73.2%) was observed in the *Gymnema* germplasm [49]. The molecular studies on *Gymnema sylvestre* [50] to characterize 18 accessions and samples of *Ceropegia* [51] have also confirmed considerable variations among those individuals. Many other members of Asclepiadaceae have also been genetically characterized by using molecular markers and or RAPD primers such as *Caralluma, Boucerosa stalagmifera* and *Bouccerosea cembellate* [52], *Calotropis* sp. [53], *Tylophora rotundifolia* [54],

UPGMA cluster analysis of all primers revealed two major clusters in this cladogram. The samples had showed 63% similarity level and 37% divergence among them. Cluster 1 was characterized by samples of *Wattakaka volubilis* and *Gymnema sylvestre* which exhibited 56% similarity and in cluster 2, samples of *Pentatropis capensis, Oxystelma esculentum and Ceropegia juncea* were clustered with 62% similarity with each other. In a study, Sanjit and Amal Kumar (2017) [55] reported that *Gymnema* sp and *Oxystelma* sp were found to fall on separate cluster. *Ceropegia arabica* was separated from *Pentatropis spiralis* at a high distance coefficient of about 1:20 [13]. Further, phylogenetic tree revealed that genus *Ceropegia* was found to be fall in separate cluster whereas *Wattakaka volubilis* and *Gymnema sylvestre* were found in present in same cluster during RAPD analysis of Apocynanceae based on trnL-F sequence [56].

Cladistic analyses using morphological [57] and molecular [58,59] data have supported the contention that Asclepiadoideae from monophyletic tribes. In a study, monophyly of the family Asclepiadaceae and of the three subfamilies Periplocoideae, Secamonoideae and Asclepiadoideae was reported by Civeyrel et al. (1998)(58). In addition, the *Asclepiads* formed a monophyletic group [60,61], and there is even some indication that the *Asclepiads* may be biphyletic. Classification of the Asclepiadaceae into sub-families, tribes and sub-tribes was based almost entirely on characters of the pollinial apparatus and overlooking the other morphological characters [62]. Based on pollinia arrangement, shape and size, erect, horizontal and pendulous were recognized to three tribes were recognized as Marsdenia, Gonolobeae and Asclepiadeae respectively [63]. Based on morphological characters, *Gymnema* sp. and *Wattakaka* sp. pollinia were found to be pendulous which were found to be in same cluster. In our study also, cluster analysis of *Gymnema sylvestre, Wattakaka volubilis, Oxystelma esculentum, Pentatropis capensis* and *Ceropegia juncea* have also shown the mixed pattern of grouping among the individuals. However, these five genera belong to the three different tribes within the subfamily of Asclepiadoideae. *Gymnema sylvestre, Oxystelma esculentum* and *Pentatropis capensis* are present in Asclepiadeae. But *Wattakaka volubilis* and *Ceropegia juncea* are present in tribes Marsdenieae and Ceropegeae respectively [64]. Our results have also proved that *Oxystelma esculentum* and *Pentatropis capensis* which are present in same cluster and showed a close relationship, belonged to the same subfamily, even of the same tribe, that is, Asclepiadeae. The position of *Gymnema sylvestre* in the separate cluster [1] consisting *Wattakaka volubilis* evidences its placement in subtribe Astephaninae [65]. This may be due to the variations influenced by insect pollination, soil conditions and other environmental factors. Species of both the clusters except *Ceropegia juncea* have shown close affinities (with genetic similarity between 52 and 58%) with each other. *Ceropegia juncea* belonged to tribe ceropegeae and therefore, in our molecular analysis, it is also present in different cluster that is subcluster 2 of cluster 2. On the other hand, convergent evolution in morphological features can lead to lumping divergent species into the same taxonomic group. Molecular systematic work by Meve and Liede (2004) [38] supported monophyly of the tribe Ceropegieae with the succulent and non-succulent genera grouped into one clade similar to the morphological analysis done by Bruyns and Forster (1991) [66]. The genus *Ceropegia*, however, was found to be paraphyletic [38,67]. The earlier morphological and molecular characterization studies are corroborated with our RAPD analysis that placed the *Ceropegia juncea* in a subcluster 2 of cluster 2. In *Ceropegia*, orientation of pollinia in pollen sac was found to be in directed upwards. Similarly, pollinia in *Ceropegia* sp. were found to be in upward direction and was placed in Ceropegieae [68]. In addition to pollen sac orientation, presence or absence of true styles and sharp constriction between stigma head and ovaries have also been used to differentiate between Ascleipideae and Ceropegieae [63]. Based on these morphological characters, *Ceropegia* sp was found to show divergence (separate cluster) when compared to *Wattakaka volubilis, Gymnema sylvestre, Pentatropis capensis* and *Oxystelma esculentum.*

The present RAPD based cluster analysis of five species of Asclepiadaceae had shown a clear picture about the position of five genera. Presence of *Wattakaka volubilis* and *Gymnema sylvestre* in a same cluster (cluster 1) revealed their close relationship with each other. Similarly, presence of *Pentatropis capensis* and *Oxystelma esculentum* in same cluster (cluster 2) showed very close relationship of them. However, the presence of *Ceropegia juncea* in separate sub cluster [2] in cluster 2 evidences that this species is so distant from other species of Asclepiadaceae (*Wattakaka volubilis, Gymnema sylvestre*, *Pentatropis capensis* and *Oxystelma esculentum*) selected in this study. Among the five species, *Ceropegia juncea* was the most diverse plant species of Asclepiadaceae. These interspecific relationships indicated that five genera belong to same sub family Asclepiadoideae but in three different tribes that is Asclepiadeae, Marsideniae and Ceropegeae. High level of similarity at intraspecific level has also shown that all the samples of each species are monophyletic. In future, if this work has been done on broader spectrum, then a complete gene pool of Asclepiadaceae can be developed. New species and varieties could be discovered. It would also help us to do an authentic and synthetic or genetic characterization of Asclepiadaceae.

5. Conclusion

In conclusion, the RAPD method successfully discriminates among all the plant species, therefore providing an easy and rapid tool for identification, conservation, and sustainable use of these plants. In future, intergeneric relationship of different plant species within the same family will enable us to attain a complete genome sequence of plants. Further, RAPD tool will be very much helpful for the taxonomist to identify the plant members and place them in the appropriate family through molecular data.

CRediT authorship contribution statement

Ponnerulan Boomibalagan: Investigation. **Sivasangari Ramya Subramanian:** Writing – review & editing. **P.E. Rajasekharan:** Validation, Software. **Souravi Karpakal:** Conceptualization, Methodology.

Uthirapandi Veeranan: Resources. **Eswaran Saminathan:** Resources. **Vijayanand Narayanan:** Writing – original draft. **Durairaj Kathiresan:** Supervision, Writing – original draft.

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