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Assessment of genetic variability among selected species of Apocynaceae

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Abstract: Family Apocynaceae is an economically important family grown as ornamental plants and many wild species have medicinal uses as well. The aim of the present study was to understand the level and pattern of genetic variability among the selected individuals of Apocynaceae. For this purpose, three species of different genera of Apocynaceae, Thevetia peruviana, *Alstonia scholaris* and C*atharanthus roseus*, were collected from Rawalpindi and Quaid-i-Azam University forest, Islamabad. To evaluate the level of polymorphism within the species and members of different species, randomly amplified polymorphic DNA (RAPD) markers were used. A series of OPC RAPD primers were used; only six primers of OPC series gave amplification. Highest genetic variation at interspecific and intraspecific levels was shown by OPC 9 and the lowest polymorphism was observed in OPC 4. The data was analyzed by using software Statistica 5.5. In total 105 monomorphic and 272 polymorphic bands were produced from all primers. Therefore, out of 322 amplified products, 26% were monomorphic and 68% were polymorphic. Low genetic diversification was observed both at intraspecific and interspecific level. At the molecular level *Alstonia scholaris* and C*atharanthus roseus* (subfamily Plumerioideae) appeared in a group and *Thevetia peruviana* (subfamily Rauvolfoideae) formed another group, confirming the classification based on morphological characters.

Key words: Apocynaceae; genetic diversity; RAPD

Introduction

The Apocynaceae or dogbane family is a family of flowering plants that includes trees, shrubs, herbs, and lianas. Many species are tall trees found in tropical rainforests, and most are from the tropics and subtropics, but some grow in tropical dry, xeric environments. There are also perennial herbs from temperate zones. Many of these plants have milky sap, and many species are poisonous if ingested (Endress & Bruyns 2000). The family, as currently recognized worldwide, includes some 1500 species, which are divided into 424 genera (Nazimuddin & Qaiser 1983).

Apocynaceae derives its economic importance from highly valued leaf anticancer alkaloids vincristine, vinblastine and antihypertensive root alkaloid ajmalicine (Srivastava & Srivastava 2007; Jaleel et al. 2006). Shazly et al. (2005) stated that ethanolic extract from the leaves of Apocynaceae contains the cardiotonic glycoside, neriifolin, which has insecticidal activity. It contains a milky sap containing a compound called Thevetin, which is being used as heart stimulant but is extremely poisonous in its natural form. Oleander extract has been used in folk medicines and there are reports that long-term use of oleander may have positive effects in patients with prostrate or breast cancer (Bose et al. 1999).

Successful management and preservation of natural populations depend on accurate assessment of genetic diversity. Genetic diversity within a population is considered to be a great importance for possible adaptation to environmental changes and consequently for long time survival of a species (Hanski & Ovaskainnen 2000). A series of techniques and genetic markers have been developed to analyze and estimate genetic diversity. Among the various markers systems, RAPDs are one of the most popular approaches (Martin & Hernandez-Bermejo 2000; Bekessy et al. 2002). The aim of this study was to use RAPD markers to identify the genetic diversity present within the members of Apocynaceae and to use this information for better management and understanding to solve the taxonomic confusion regarding the classification of Apocynaceae.

Material and methods

Sample collection

Plants of three different genus of the family *Apocynaceae* (*Thevetia, Alstonia*, and *Catharanthus*) were collected from Rawalpindi and Quaid-i-Azam University forest, Islamabad

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in March. Five plants for each genus were picked to evaluate variation within species. Young leaves were collected and stored at 4℃ in sealed plastic bags till further process.

DNA extraction
In case of *Thevetia peruviana,* total genomic DNA was extracted from fresh leaves by CTAB method (Richards 1997) with few modifications, while *Alstonia and Catharanthus* genomic DNA was isolated by using liquid nitrogen due to the presence of high contents of secondary metabolites. Finally, DNA was dissolved in 0.1× TE (Tris-Ethylene Diamine Tetra Acetic Acid) buffer (pH 8.0). The DNA was quantified by spectrophotometer and purity was checked by running it on 1% agarose gel prepared in 0.5× TAE buffer (Tris Acetate Ethylene Diamine Tetra Acetic Acid).

PCR Amplification
A set of primers from OPC series was used for amplification in RAPD analysis. PCR conditions used for amplification were pre PCR denaturation at 94℃ for 1 min followed by 44 cycles of denaturation at 94℃ for 30 sec, annealing at 40℃ for 1 min and a final period of extension at 72℃ for 2 min. Final cycle was the same except extension for 7 min at 72℃. After PCR, the contents were held at 4℃ till use. About 25 µL of the PCR mixture contained 50 pmol of primer, 12.5 µL of 2 × PCR master mixes (Fermentas), 10.5 µL of nuclease free water and 50 ng of genomic DNA.

Electrophoresis of the PCR product
Amplification products were run on 1.5% agarose gel prepared in 0.5× TAE buffer, stained with ethidium bromide and visualized under UV light. Gel documentation was carried out by Dolphin Doc Plus gel documentation system (Wealtec).

Data scoring and analysis
The presence of a particular band was scored as 1 and absence as 0. Bands with same mobility were treated as identical fragments. The positions of PCR bands were compared with molecular weight standards (Fermentas). Data analysis was performed using the software package Statistica 5.5. After processing the gel images, all pair-wise similarity values were calculated with a program.

Results and discussion

Isolation of Genomic DNA
Thevetia peruviana, Alstonia scholaris and *Catharanthus roseus* are medicinal and aromatic plants and probably have large amount of secondary metabolites and essential oil. Molecular studies dealing with medicinal and aromatic plants are rare in comparison with other cultivated plants due to the presence of medicinal compounds, which inhibit DNA amplification in PCR reaction (Khanuja et al. 1999; Mizukami & Okabe 1999). Protocol by Richards (1997) gave good quality DNA of *Thevetia peruviana*, while liquid nitrogen was used for isolation of genomic DNA of *Alstonia scholaris* and *Catharanthus roseus*. In PCR high quality of DNA is required for amplification purposes, therefore the quantity and quality of DNA was confirmed by spectrophotometer and agarose gel electrophoresis (Table 1). Using this genomic DNA as a template, amplification was carried out by using different RAPD primers of OPC series.

Table 1. Quantification of DNA samples by spectrophotometer.

Sr. No.	Species	Samples Code	Ratio A260:A280
1	*Thevetia peruviana*	THEV1	1.67
2	*Thevetia peruviana*	THEV2	1.83
3	*Thevetia peruviana*	THEV3	1.61
4	*Thevetia peruviana*	THEV4	1.89
5	*Thevetia peruviana*	THEV5	1.71
6	*Alstonia scholaris*	ALST1	1.99
7	*Alstonia scholaris*	ALST2	1.90
8	*Alstonia scholaris*	ALST3	1.88
9	*Alstonia scholaris*	ALST4	1.80
10	*Alstonia scholaris*	ALST5	1.92
11	*Catharanthus roseus*	CATHA1	1.75
12	*Catharanthus roseus*	CATHA2	1.62
13	*Catharanthus roseus*	CATHA3	1.69
14	*Catharanthus roseus*	CATHA4	1.56
15	*Catharanthus roseus*	CATHA5	1.72

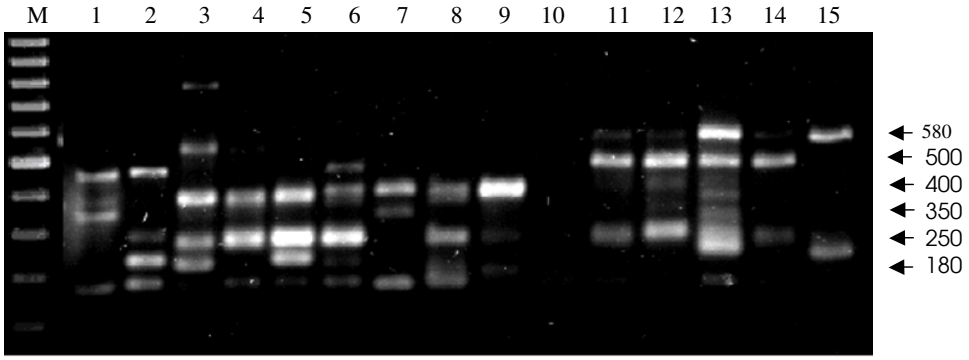
Out of the ten different OPC primers, six have given results. The amplified products of these six primers were run on 1.5% agarose in 0.5× TAE.

RAPD analysis
After screening it was observed that a total of 402 bands have been generated by six RAPD primers across the fifteen samples of three different genera (*Thevetia peruviana, Alstonia scholaris* and *Catharanthus roseus*) of family Apocynaceae. The molecular weights of the amplified products were in the range of 200bp to 1100bp. Out of the 402 bands, 272 were found to be polymorphic (68%) and 105 were monomorphic. The results showed that RAPDs are an abundant source of polymorphic markers in Apocynaceae. The optimized RAPD-PCR protocol produced highly reproducible banding patterns. The banding pattern obtained showed a high genetic variation among genera and individuals of same species. OPC4 generated the maximum of 74 numbers of bands whereas; OPC8 produced the minimum number (58). High level of polymorphism was generated by OPC9 (94.44%), while the minimum number of polymorphic bands were produced by OPC6 among the samples tested. Further, it was observed that OPC5, OPC6 and OPC8 have produced unique bands of different sizes (Table 2), which could be used as molecular markers for individual identification (Fig.1). RAPD technique is considered as a very sensitive method of screening of molecular markers randomly distributed throughout the genome (Subudhi & Huang 1999).

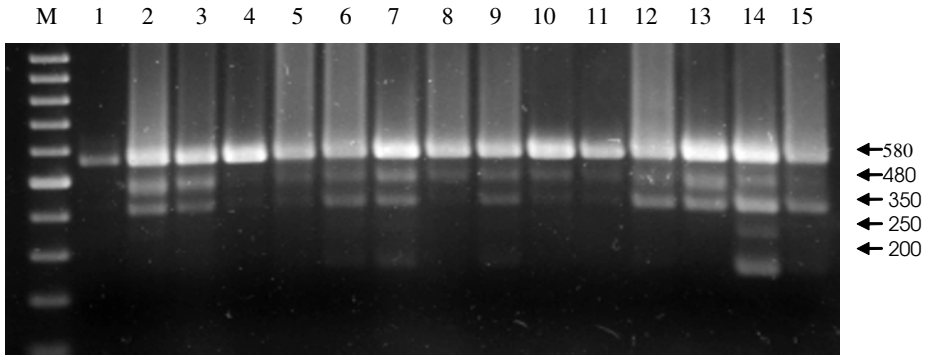
Statistical analysis
Although the bands varied in size, the results from each RAPD product were assumed to represent a single locus and data was scored as the presence (1) or absence (0) of a DNA band. Only those fragments that amplified consistently were considered for analysis. UPGMA cluster analysis divided the 15 individuals of Apocynaceae into two main clusters at 0.35 genetic distances and had 65% similarity. Cluster 1 had samples of *Alstonia schol-*

Table 2. Percentage polymorphism generated by different RAPD primers of OPC series.

S.No.	Primers	Monomorphic bands	Polymorphic bands	Unique bands	Rare bands	Total bands bands	Percentage polymorphism
1	OPC2	15	50	0	5	70	71.4
2	OPC4	30	41	0	3	74	55.4
3	OPC5	15	48	2	3	68	69
4	OPC6	30	27	1	2	60	50
5	OPC8	15	38	3	2	58	66
6	OPC9	0	68	0	4	72	94.44
Total		105	272	6	19	402	68



Banding pattern produced by OPC2



Banding pattern produced by OPC6

Fig. 1. PCR amplification with OPC2 and OPC6. Lane 1: M: DNA ladder marker, lane 2 to 6: *Thevetia peruviana* (1–5) DNA, lane 7 to 11: *Alstonia scholaris* (1–5) DNA, lane 12 to 16: *Catharanthus roseus* (1–5) DNA.

aris and *Cathranthus roseus* and further showed divergence at 0.32 genetic distances into two subclusters. Sub cluster1 had samples of *Catharanthus roseus*, while sub cluster 2 had samples of *Alstonia scholaris*. Samples of *Thevetia peruviana* grouped into cluster 2 which belongs to another subfamily Rauvolfoideae (Fig. 2).

During the present study, a high degree of polymorphism was observed at intrageneric level (68%). The analysis of the degree of polymorphism is important for the conservation of populations in isolated reserve areas. Isolation of populations can influence genetic drift and can limit intra-specific diversity. Other influential factors include the extraction of non-lumber products and fragmentation. Fragmentation disrupts the natural continuity of habitats and may cause a decrease in gene flow between populations. This causes a loss of genetic variability and limits species evolution (Barrett & Kohn, 1991).

Based on the results obtained, it was concluded that family Apocynaceae has shown genetic diversity among its three different genera with six RAPD primers of OPC series. Further, it was observed that low genetic diversification is present at interspecifc and intraspecific levels. Moreover, *Alstonia scholaris* and *Catharanthus roseus* appeared in one cluster belonging to subfamily Plumerioideae, while all samples of *Thevetia peruviana* (subfamily Rauvolfoideae) grouped together in a second cluster. These interspecific relationships indicated that the three genera belong to two subfamilies i.e. Rauvolfoideae and Plumerioideae. High level of similarity at intraspecific level has also shown that all the samples of each species are monophyletic. It can

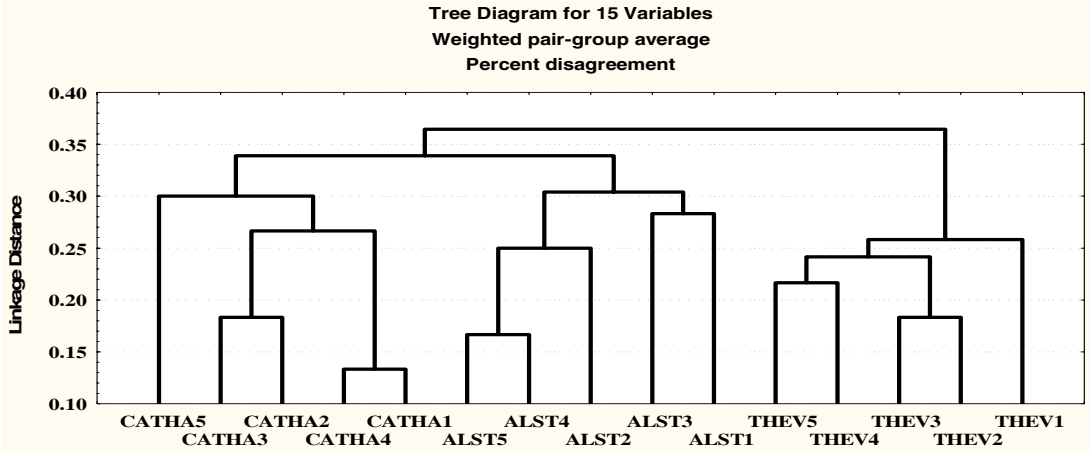


Fig. 2. Genetic diversity found among each sample of three genera. *Thevetia peruviana* (THEV1–THEV5), *Alstonia scholaris* (ALST1–ALST5), *Catharanthus roseus* (CATHA1–CATHA5 with all six primers.

be inferred from the present investigation that RAPD technique is a useful tool for the analysis of genetic diversity among the genera of Apocynaceae. RAPD can produce a large set of markers, which can be used for the evaluation of genetic variation both among and within-species.

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