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COMPARATIVE ASSESSMENT OF GENETIC VARIABILITY IN *CRYPTOLEPIS BUCHANANII*, *TYLOPHORA HIRSUTA* AND *WATTAKAKA VOLUBILIS*

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Abstract

Asclepiadaceae is an economically important family with great medicinal value. However, very little work has been carried out on the genetic variability of Asclepiadaceae members especially on some medicinally important species like *Tylophora hirsuta*, *Wattakaka volubilis* and *Cryptolepis buchananii*. Keeping in view the importance of these species, a study was designed to explore the genetic diversity of these 3 species of Asclepiadaceae and the plant material was collected from Quaid-i-Azam university campus, Islamabad. To assess the genetic variability and polymorphism among these species, randomly amplified polymorphic DNA (RAPD) markers were used. Sixty RAPD primers from OPA, OPC, OPF and OPG series were used; only 8 primers of OPC series gave amplification. Maximum polymorphism at interspecific and intraspecific levels was shown by OPC9 and minimum polymorphism was observed in OPC5. The data was analyzed using NTSYS software pc version 2.02. Low genetic diversification was observed at intraspecific and interspecific level. Moreover, during cluster analysis *Tylophora hirsuta* and *Wattakaka volubilis* were found to be present in the same cluster showing a close relationship whereas *Cryptolepis buchananii* appeared in a separate cluster.

Introduction

A family Asclepiadaceae with 175-180 genera and 2000 species is distributed mainly in the tropical and subtropical regions of the world. In Pakistan, it is represented by 23 genera and 41 species. Members of Asclepiadaceae are distributed in paleotropical regions. It is distributed throughout India, Nepal, Cylon, Malaysia, South China, tropical Asia and Africa. In Pakistan, they are distributed in North Punjab, Azad Kashmir, Hazara, Rawalpindi, Attock and salt range Ali (1983).

The members of the family Asclepiadaceae have been used for the treatment of various diseases. The alcoholic extract of *Wattakaka volubilis* is widely used in India as a traditional medicine as an antidote in snakebites, boils and abscesses (Reddy, 2003). This plant also contains potential hypoglycemic agents, which are used in anti-diabetic therapy. Leaf powder is used usually for this purpose (Ayyanar *et al*., 2008). *Cryptolepis buchananii* is a very useful plant because of its multiple uses as a traditional medicine, such as anti-diarrhoeal, anti-bacterial, anti-ulcerative, anti-inflammatory, blood purifier and for lactation in women (Bhakuni *et al*., 1969). In Thailand, the alcoholic extract of stem of this plant has been commonly used for the treatment of inflammatory conditions such as arthritis, and muscle and joint pain (Panthong *et al*., 1986; Laupattarakasem *et al.,* 2003). An additional important feature of *Cryptolepis buchananii* is its low toxicity (Laupattarakasem *et al.,* 2006). *Tylophora indica* has been traditionally used for the treatment of bronchial asthma, dysentery, inflammation, jaundice and to induce emesis. Preliminary phytochemical investigations showed the presence of alkaloids, carbohydrates, saponins, steroids and triterpenes (Patel *et al.*, 2008).

The infrafamilial classification of *Asclepiadaceae* was proposed by Brown (1810) and further description was provided by Liede & Albers (1994). Based on these reports the family Asclepiadaceae can be divided into three subfamilies of which the Periplocoideae and Secamonoideae are monotribal and Asclepiadoideae usually comprises three tribes. Later on, Venter & Verhoeven (1997) recognized three tribes in Periplocoideae. The monophyly of the Asclepiadaceae is uncertain as reported by Sennblad & Bremer (1996). The subfamily *Periplocoideae* was found to be monophyletic and did not form a monophyletic group with the other two subfamilies (Liede & Albers, 1994), whereas in the study of Civeyrel *et al.* (1998) the three subfamilies did not form a monophyletic group. The traditional concept of classification of *Asclepiadaceae* receives good support (Sennblad & Bremer, 1996; Civeyrel *et al*., 1998; Potgieter & Albert, 2001). In the present account, the *Asclepiadaceae* is treated as a family, which according to Schumann (1895), Bruyns & Forster (1991) and Liede & Albers (1994) is subdivided into three subfamilies *Periplocoideae, Secamonoideae* and *Asclepiadoideae*. Although the classification of Endress & Bruyns (2000) is a major advancement in making the classification matching with current best estimates of phylogeny, problematic cases still need revision. In which they include all three subfamilies into the *Apocynaceae* and to abandon the *Asclepiadaceae.* Furthermore, for many of the recognized tribes, taxon sampling for molecular data is weak and the morphological homologies in these groups are difficult to interpret (Endress & Bruyns, 2000). So the most widely accepted suprageneric system of the *Asclepiadaceae* by Bruyns & Forster (1991), which largely relies on morphological data is based on the classification of Brown (1810) and Schumann (1895). Plastid gene *matK* was used to resolve the complex relationships of *Apocynaceae* and *Asclepiadaceae.* Molecular and morphological data had shown without any ambiguity that *Apocynaceae/ Asclepiadaceae* form a monophyletic group (Civeyrel *et al.,* 1998). Meve & Schumann (2007) on the basis of molecular study of *Ceropegia* taxa reported that results are incongruent with current taxonomy. In the absence of adequate

morphological, anatomical, or karyological characters supporting a taxonomic reclassification of the genus in accordance with the retrieved clades of the phylogenetic analysis, it was proposed that the current taxonomy be maintained. For further investigation the present study was conducted to check the taxonomic status at molecular level. As very little work has been done on the molecular aspect of *Tylophora hirsuta, Cryptolepis buchananii* and *Wattakaka volubilis,* the need was felt to explore and study the genetic variations of these plants by using RAPD markers.

Material and Methods

Sample collection: Plants of three different genera of *Asclepiadaceae (Tylophora, Cryptolepis, Wattakaka*) were collected from Quaid-i-Azam University campus Islamabad. Area lies at latitudes 33° 49' North and longitudes 73° 10' East with altitudes ranging from 457 to 610 meters. It is quite moderate in case of its weather. Five samples (young leaves) of each species i.e., *Tylophora hirsuta*, *Cryptolepis buchananii* and *Wattakaka volubilis* were collected and stored at 4˚C in sealed plastic bags.

DNA extraction and DNA quality: Total genomic DNA was extracted from fresh leaves by CTAB method (Richards, 1997) with few modifications. The DNA was quantified by spectrophotometer and its quality was checked by running it on 1% agarose gel prepared in 0.5X TAE buffer (Tris Acetate Ethylene Diamine Tetra Acetic Acid).

Screening of primers: Sixty primers of different RAPD series (Gene link, 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. Ten random primers of OPC series (OPC 01 to OPC 10) were tested for RAPD analysis. Scoreable amplification profiles were given by all above mentioned primers of OPC 01 to OPC 10 except OPC 03 and OPC 10.

Polymerase chain reaction (PCR) conditions: Different PCR conditions were used for the optimization of amplification, however the best suitable conditions were as follows; initial denaturation at 94ºC for 1 minute followed by 44 cycles of denaturation at 94 ºC for 30 seconds, annealing at 40ºC for 1 minute and extension at 72ºC for 2 minutes. Final cycle was same except extension for 7 minutes at 72ºC. After PCR, contents were held at 4ºC till use. The genomic DNA was used for PCR amplification reactions in a 25 µl reaction mixture containing 25-50 ng/µl DNA, 25 pmol primer, 12.5 µl 2X PCR master mix (Fermentas) and 10.5 µl of water (Fermentas) by using gradient thermal cycler (Labnet, multigene). Amplified products were loaded on 1.5% agarose gel prepared in 0.5X TAE buffer. The gel was allowed to run for two hours at constant voltage followed by staining in ethidium bromide (0.1 mg/10 ml). Gel documentation was carried out by Dolphin Doc Plus gel documentation system (Wealtech). The size of each band was estimated by using 100 bp DNA ladder plus (Fermentas).

Data scoring and analysis: Bands on a gel were recorded as present (1) or absent (0). Cluster analysis was carried out based on similarities within class and dissimilarities among different classes i.e., samples showing good correlation have been placed in same group. All monomorphic and polymorphic bands were scored and included in the analysis. Presence or absence of unique and rare bands was used to generate genetic similarity coefficients and then similarity coefficients were used to construct dendrogram by computer program Numerical Taxonomy and Multivariate Analysis System (NTSYS) pc version 2.20 (Rohlf, 2002).

Results and Discussion

DNA isolation and polymerase chain reaction: Genomic DNA was isolated from leaves of *Tylophora hirsuta, Cryptolepis buchananii* and *Wattakaka volubilis*. Isolation of good quality DNA is the crucial step before applying molecular markers. Sometimes secondary metabolites and impurities in genomic DNA cause hindrance in the amplification reactions. Isolated genomic DNA was run on 1% agarose gel after treatment with RNase A for determining the quality of DNA and spectrophotometric analysis was carried out for the estimation of DNA quantity. By using this genomic DNA as a template, amplification was carried out by different RAPD primers. Amplified products were confirmed by running PCR products on 1.5% agarose in 0.5X TAE at constant voltage.

Selection of molecular marker: Various types of molecular markers have been utilized to evaluate DNA polymorphism and are generally classified as hybridization-based markers and PCR based markers (Saiki *et al.,* 1985). RAPD is one of the PCR based marker system which has been used widely in different studies (Nazar & Mahmood, 2011; Mahmood *et al.,* 2011a; Mahmood *et al.,* 2011b; Shinwari *et al.,* 2011; Mahmood *et al.,* 2010a; Mahmood *et al.,* 2010b) and was used in this study. Primer directs amplification of several discrete loci in the genome, making the reaction useful for efficient screening of nucleotide sequence polymorphism among individuals (Tingey *et al.,* 1993). The reproducibility of the RAPD technique can be influenced by various factors, such as primer sequence, template quality and quantity, the type of PCR machine and polymerase concentration (Hernendez *et al.,* 1999).

RAPD profile: Only eight primers from PRC series were shown to produce amplification from DNA samples of three different species of *Asclepiadaceae* representing three different genera among which six primers produced reproducible results. A total of 322 clear bands were amplified out of which 78 were polymorphic and showed 24.22% polymorphism (Table 1). Earlier, by using RAPD analysis considerable genetic variation (73.2%) was observed in the *Gymnema* germplasm collection from Kerala (Nair & Keshavachandran, 2006).

Table 1. Number of bands generated and percentage of polymorphism as revealed by RAPD primers.

S. No.	Primers	Total bands	Monomorphic bands	Polymorphic bands	Rare bands	Unique bands	Polymorphism %
1.	OPC4	75	75	0	0	0	0
2.	OPC-5	63	30	20	12	1	31%
3.	OPC-6	49	20	17	8	4	34%
4.	OPC7	45	45	0	0	0	0
5.	OPC8	42	10	18	6	8	42%
6.	OPC9	48	10	23	8	1	47%
	All primers	322	190	78	34	14	24%

The number of amplicons generated varied from 75 (OPC 04) to 42 (OPC 08) and represented molecular weight of 200 bp to 1200 bp. Maximum number of bands were produced by OPC 04 while minimum number of bands were produced by OPC 08 and OPC 09. However, no polymorphic band was observed in OPC 04 and OPC 07, therefore have shown 100 % similarity. OPC 05, OPC 06, OPC 08 and OPC 09 generated unique fragments of different molecular sizes which could be used as molecular markers for individual identification (Fig. 1). Polymorphism was observed within the species and among the species collected from same habitat. In an earlier study RAPD markers were used to analyze genotypic changes for evaluating variations in *in vitro* regenerated *Tylophora indica* and revealed no variation (Leroy *et al.,* 2000).

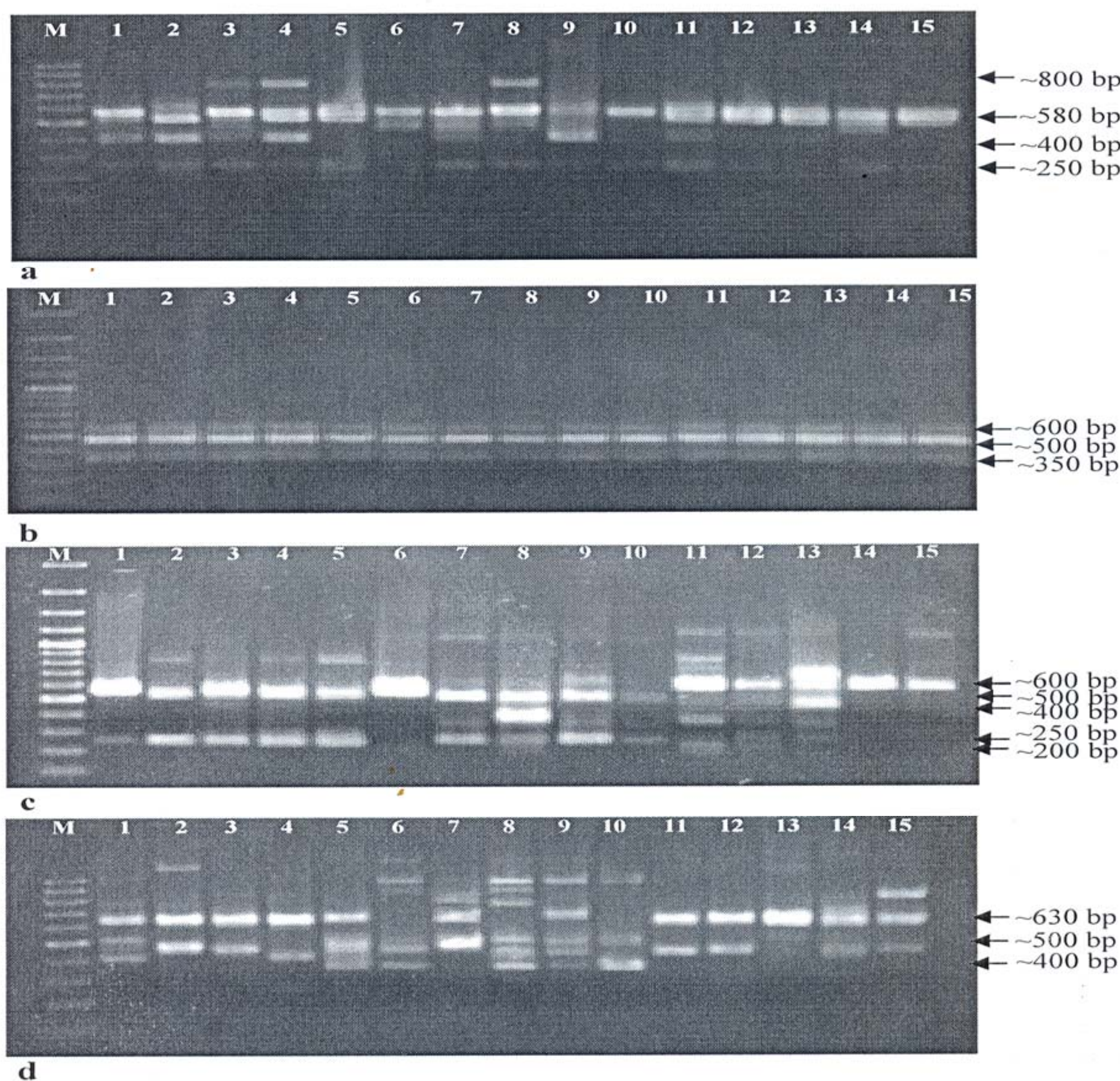

Fig. 1. Banding pattern produced by OPC 06 (a), OPC 07 (b), OPC 08 (c) and OPC 09 (d). M: marker (100 bp ladder plus, Fermentas for a, b, c and 100 bp ladder, Fermentas for d), *Tylophora hirsuta* (sample 1-5), *Cryptolepis buchananii* (sample 6-10) and *Wattakaka volubilis* (sample 11-15).

Cluster analysis: Similarity indices were developed on the basis of amplified products of 6 RAPD primers with samples of 3 different species *viz. Tylophora hirsuta*, *Cryptolepis buchananii* and *Wattakaka volubilis*. The range of genetic similarity values was observed, which was in the range of 0.42 to 0.85 with the mean of 0.63 (Table 2).

UPGMA cluster analysis of all primers revealed two major clusters in the cladogram. Data from six primers of OPC series were analyzed in the cluster analysis and samples showed 63 % similarity level and 37 % divergence. Cluster 1 was characterized by 10 samples *viz.* 1, 2, 3, 4, 5, 11, 12, 13, 14 and 15. Cluster 1 further showed divergence at 70 % similarity level and showed two subclusters. All samples of *Tylophora hirsuta* (2, 3, 4 and 5) excluding sample 1 were clustered together in subcluster 1 and all samples of *Wattakaka volubilis* (11, 12, 13, 14 and 15) appeared in subcluster 2. Only sample number 1 of *Tylophora hirsuta* showed genetic relatedness with samples of *Wattakaka volubilis* and appeared in subcluster 2. *Cryptolepis buchananii* showed clear demarcation and appeared in a separate cluster mentioned in figure as cluster 2 (Fig. 2).

Table 2. Genetic Similarity coefficients of three species (*Tylophora hirsuta, Cryptolepis buchananii* and *Wattakaka volubilis*) based on Euclidean Distance.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1.	1.00														
2.	0.73	1.00													
3.	0.76	0.83	1.00												
4.	0.78	0.80	0.78	1.00											
5.	0.69	0.85	0.78	0.80	1.00										
6.	0.57	0.50	0.61	0.50	0.64	1.00									
7.	0.71	0.69	0.80	0.64	0.69	0.66	1.00								
8.	0.50	0.42	0.59	0.47	0.57	0.73	0.69	1.00							
9.	0.66	0.59	0.66	0.59	0.69	0.71	0.76	0.78	1.00						
10.	0.69	0.61	0.64	0.66	0.76	0.73	0.73	0.76	0.83	1.00					
11.	0.69	0.66	0.73	0.57	0.61	0.69	0.73	0.57	0.64	0.57	1.00				
12.	0.80	0.69	0.71	0.64	0.69	0.66	0.76	0.64	0.66	0.69	0.78	1.00			
13.	0.71	0.69	0.76	0.64	0.64	0.66	0.71	0.54	0.66	0.64	0.83	0.71	1.00		
14.	0.78	0.76	0.78	0.71	0.76	0.73	0.73	0.52	0.59	0.61	0.76	0.83	0.69	1.00	
15.	0.71	0.64	0.71	0.59	0.69	0.71	0.71	0.50	0.57	0.59	0.78	0.80	0.76	0.83	1.00

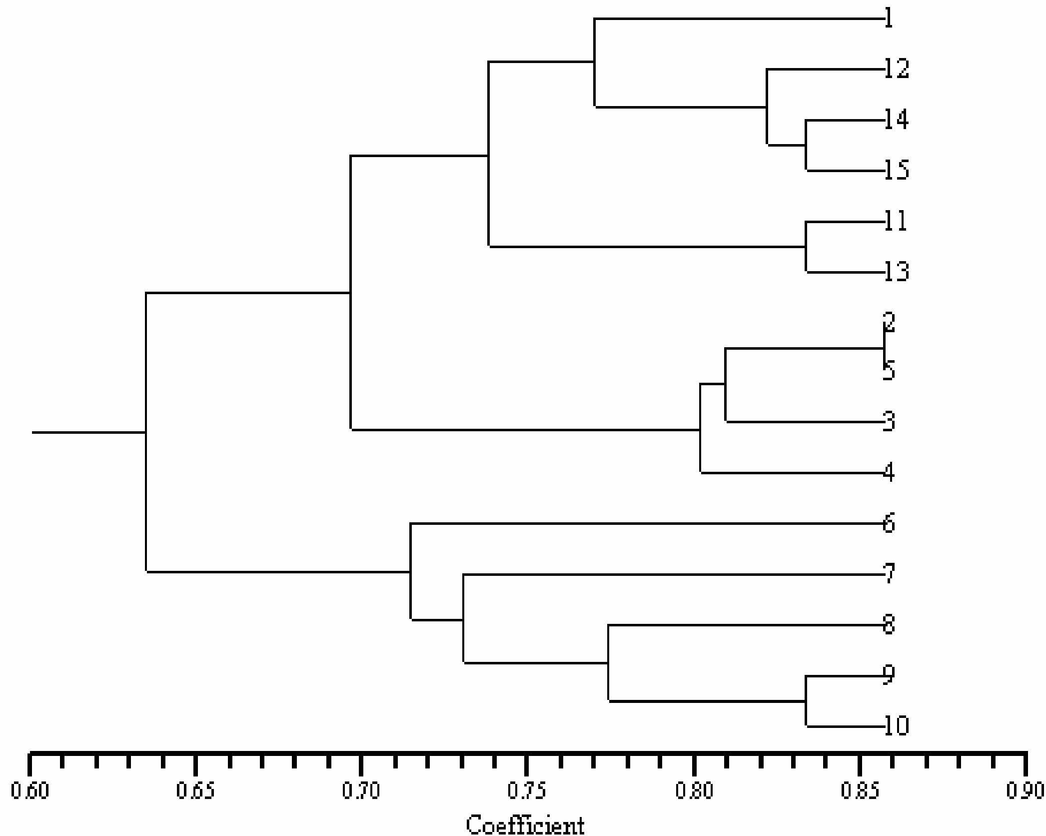

Fig. 2. Dedrogram producd by amplified products of all primers. The number corresponds to the species as follows: *Tylophora hirsuta*: (samples 1, 2, 3, 4, 5), *Cryptolepis buchananii*: (samples 6, 7, 8, 9, 10), *Wattakaka volubilis*: (samples 11, 12, 13, 14, 15).

Cluster analysis of all primers has shown that genetic diversity is present within the species and among different species. Species are the units of ecological diversity and alleles are the units of genetic diversity. Samples of different species simply harbor more distinct alleles than samples of the same species. Weighting of ecological measures of species diversity on the basis of their genetic distance has been considered by several authors (May, 1990; Humphries *et al.*, 1995). These results indicate that these species have a very close affinity with each other. These three genera belong to two different subfamiles within *Asclepiadaceae*. It was evident that members of *Asclepiadaceae* are insect pollinated, which could have affected the evolutionary processes to produce variability within the constituent taxa. Different studies had revealed that tribes *Marsdenieae* and *Asclepiadeae* are the most prominently *Diptera* serviced groups (Ollerton & Liede, 1997). *Tylophora hirsuta* and *Wattakaka volubilis* are belong to subfamily *Asclepiadoideae* and *Cryptolepis buchananii* is a member of subfamily *Periplocoideae.* Our results have also proved that *Tylophora hirsuta* and *Wattakaka volubilis*, which are present in same cluster and showed a close relationship, belonged to a same subfamily, even of the same tribe i.e. *Marsdenieae*. Species of this cluster have shown close affinities with each other. While *Cryptolepis buchananii* belongs to different subfamily i.e. *Periplocoideae*, and in our analysis, it is also present in different cluster.

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