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Full Length Research Paper

Genetic diversity of selected Apocynaceae species based on chloroplast gene *rps*11

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Apocynaceae is an important family due to its credible therapeutic importance and it is widely distributed in tropics and subtropics. Some species of Apocynaceae have been randomly chosen from different regions of Pakistan for the present study. The main objective was to analyze genetic diversity among seven species using cleaved amplified polymorphic sequences (CAPS) technique on a plastid gene encoding ribosomal protein of smaller subunit 11 (*rps*11). For this purpose, DNA was extracted from young leaves and with the help of a pair of primer, *rps*11 gene was amplified and seven restriction enzymes namely: *Tsc*AI, *Scr*FI, *Dpn*I, *BsiKHA*I, *Mse*I, *Hin*fI, *Bse*GI were used to digest the amplified *rps*11 gene. The results produced were in the form of bands on gels revealing the length of fragments produced after cutting with restriction enzymes. The digested fragments were found to produce monomorphic bands whereas some polymorphic bands were also observed. On the basis of restricted fragments, phylogenetic tree was prepared depicting different number of clusters with varied level of similarity coefficients. It was observed that the species have shown mixed pattern and closely related species appeared at higher genetic distances. It can be concluded from the results that CAPS on *rps*11 gene could be used as a useful source for phylogenetic analysis among the family Apocynaceae.

Key words: Apocynaceae, chloroplast, cleaved amplified polymorphic sequences (CAPS), phylogenetic analysis.

INTRODUCTION

Plants play very critical roles for the sustainability of life on earth. Among so many important functions, plants are being used as esthetic, medicinal, food, industrial products, recreation, air quality, water quality, erosion, climate, fish and wild life habitat and ecosystem. About 90% of the world's food comes from 20 species of plants. Besides, 3000 species are supporting the food to world population. Plant species known on earth are approximately 4, 22,127 (Hasan et al., 2007). Pakistan has uniqueness in ecological diversity and adopts a bowel of biodiversity due to diverse climatic condition and geography. In Pakistan, six thousand flowering species has already been identified (Shinwari et al., 2006). Among these plants, Apocynaceae is an important family due to its credible economical importance. Pakistan lies at the North of the equator and have considerable variety of genera of Apocynaceae. Mainly the members of Apocynaceae are present in North Punjab, Azad Kashmir, Hazara, Rawalpindi, Attock and salt range of Pakistan (Ali, 1983).

The plants of Apocynaceae are economically important for ornamental purposes as well as for having medicinal properties like pungent, emetic, purgative and diaphoretic. The latex is usually acrid and bitter, but occasionally it is used as blend in milk, as in the case of *Gymnema lactiferum*, the cow-plant of Ceylon (Chopra et al., 1956). It is a tradition to use different plants in daily diet to maintain the health and nutritional level. One such species, *Caralluma tuberculata* is used as a vegetable and its roots and stem extracts are being used for curing stomach ailments. Another species *Caralluma edulis* is helpful in blood related diseases and it has been used as vegetable (Ali, 1983). The bulby root of yet another important plant *Ceropegia bulbosa* is also being used as vegetable in the sub-continent. Some members of Asclepiadoideae are being used commercially, such as Calotropis has been domestically used to fill

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Table 1. List of species of family Apocynaceae from different sites.

S/N	Species name	Site name	Longitude and latitude
1	*Hoya longifolia*	Chakothi (Azad Kashmir)	73° 75′ N and 33° 36′ E
2	*Wattakaka volubilis*	Islamabad	33° 42′ N and 73°10′ E
3	*Telosma cordata*	Chakothi (Azad Kashmir)	73° 75′ N and 33° 36′ E
4	*Caralluma edulis*	Mianwali	32° 38′ N and 71°28′ E
5	*Caralluma tuberculata*	Mianwali	32° 38′ N and 71° 28′ E
6	*Tylophora hirsuta*	Islamabad	33° 42′ N and 73° 10′ E
7	*Cryptolepis buchananii*	Islamabad	33° 42′ N and 73° 10′ E

mattresses and pillows to make them stuffy and soft and *Marsdenia tinctoria* yields a dye to be used in textile industry (Ali, 1983). In soap industry, *Tuberlosa* L. has been used for making liquid soap and it has some semi drying oil in it and has shown some promising role in textile industry (Pobedimova, 1952). The members of Asclepiadoideae family have also been used for horticultural purposes. A wide range of ornamental plants are members of this subfamily like *Asclepia, Caralluma, Ceropegia, Dischidi, Hoya, Stapelia,* and *Stemphanotis* (Chopra et al., 1956).

Molecular marker research in plant unlocks the genetic potential for assessing the beneficial and desirable traits. Molecular marker can identify the location of desired traits by exploiting the polymorphism. Several PCR-based or non-PCR based molecular markers are available to access and exploit the genetic diversity. There are different types of molecular markers, which are in constant usage.

Some of them are, allele specific associated primers (ASAP) (Gu et al., 1995), amplified fragment length polymorphism (AFLP) (Vos et al., 1995), arbitrarily primed polymerase chain reaction (AP-PCR) (Welsh and McClelland, 1990), expressed sequence tags (EST) (Adams et al., 1993), restriction fragment length polymorphism (RFLP) (Botstein et al., 1980), cleaved amplified polymorphic sequence (CAPS) (Akopyanz et al., 1992), random amplified microsatellites polymorphism (RAMP) (Wu et al., 1994), inter-simple sequence repeat (ISSR) (Zietkiewicz et al., 1994), random amplified polymorphic DNA (RAPD) (Williams et al., 1990), simple sequence repeats (SSR) (Akkaya et al., 1992), variable number tandem repeats (VNTR) (Nakamura et al., 1987) and single nucleotide polymorphism (SNP) (Jordan and Humphries, 1994). In fact, these different types of molecular markers have been classified on the basis of their differences in principles, methodologies and applications but yet no marker is available to fulfill all the requirements of the researchers. Among these molecular markers, CAPS has several advantages as it is more valuable genetic marker for genetic mapping studies than non-functional sequences based markers and the primers for CAPS are being developed from ESTs. Moreover, CAPS markers are inherited in co-dominant way, easier to use and less time consuming (Matsumoto and Tsumura, 2004).

In the present study, cleaved amplified polymorphic sequence (CAPS) has been used to evaluate the genetic diversity of randomly selected species of Apocynaceae, belonging to subfamily Asclepiadoideae (*Hoya longifolia*, *Wattakaka volubilis*, *Telosma cordata*, *Caralluma edulis*, *Caralluma tuberculata*, *Tylophora hirsuta*) and subfamily Periplocoideae (*Cryptolepis buchananii*) on the basis of chloroplast gene encoding ribosomal protein of smaller subunit 11 (*rps*11). The data produced has been analyzed for establishing the phylogenetic relationship among seven different species of Apocynaceae.

MATERIALS AND METHODS

Collection of plant material

The selected species of Apocynaceae were collected from different regions of Pakistan (Table 1) and species were identified with the help of available information and voucher numbers in the National Herbarium of Pakistan, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan. Young leaves of each species were collected for DNA isolation.

DNA isolation and quantification

For extraction of total genomic DNA, CTAB (Cetyl Trimethyl Ammonium Bromide) method was used with few modifications (Nazar and Mahmood, 2011; Mahmood et al., 2010) and the DNA samples were checked on 1% agarose gel. After staining in ethidium bromide the gel was visualized in a gel documentation system (Dolphin-Doc ^{Plus}, Wealtech). The concentration of the DNA was measured by spectrophotometer (Smart SpecTM Plus) at 260 nm and high quality DNA was used for the amplification purposes.

Primers designing and amplification of *rps*11 gene

A pair of primer was designed from tobacco chloroplast genome (Accession # Z00044.2) available in Genbank for the amplification of *rps*11 gene, using the available online program Primer ‘3’. The sequence of the primers is as follows:

*rps*11 F: 5’ TGGCAAAAGCTATACCGAAAA 3’
*rps*11 R: 5’ TTCGGAGGTCTACAGCCATT 3’

Genomic DNA was used as template for the amplification of *rps*11

gene. The conditions used for PCR amplification were pre PCR denaturation at 94°C for five minutes followed by 35 cycles of

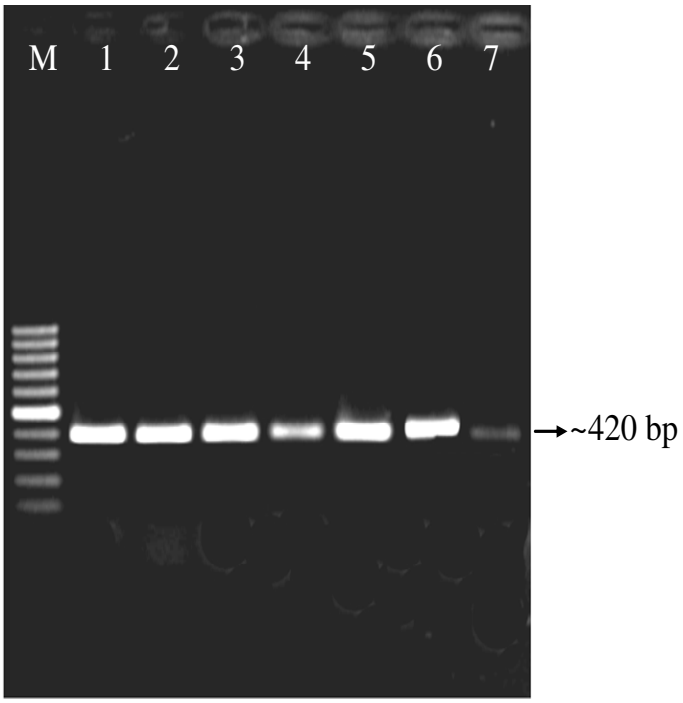

Figure 1. Amplification of *rps*11 gene from different species of Apocynaceae; M: 100 bp ladder (Fermentas), 1: *Hoya longifolia*, 2: *Wattakaka volubilis*, 3: *Telosma cordata*, 4: *Caralluma edulis*, 5: *Caralluma tuberculata*, 6: *Tylophora hirsuta*, 7: *Cryptolepis buchananii*.

denaturation at 94°C for one minute, annealing at 60°C for one minute which was followed by extension at 72°C for one minute. At the end, a final cycle was programmed which was same as the previous ones, except that it was extended for twenty minutes at 72°C. The PCR reaction mixture contents were held at 4°C. A 25 µl PCR reaction contains 50 pM of each primer, 2.5 µl of 10X *Taq* buffer, 1.5 µl of 2 mM dNTPs, 1.5 µl of 25 mM $MgCl_2$ and 5U of *Taq* polymerase (MBI Fermentas) was used. The amplification conditions were optimized using gradient PCR (Multigene, Labnet) and the amplified products were analyzed by running them on 1.5% agarose gel.

Cleaved amplified polymorphic sequences (CAPS)

Mapping of restriction enzymes on amplified rps11 gene

The sites of restriction enzymes were theoretically mapped on *rps*11 gene sequence from tobacco by using the online tool NEBcutter (http://tools.neb.com/NEBcutter2). The observed restriction pattern was applied practically on the amplified *rps*11 gene from seven species of Apocynaceae. In total, seven restriction enzymes were chosen to digest the amplified *rps*11 gene. The names of the restriction enzymes used for digestion of the *rps*11 gene from selected species were *Tsc*AI, *Scr*FI, *Dpn*I, *Bsi*HKAI, *Mse*I, *Hin*fI and *Bse*GI.

Poly acryl amide gel electrophoresis (PAGE)

The digested samples were run on 12% PAGE (BioRad) in 0.5 X TBE (Tris Borate EDTA) buffer and the gel was then stained with silver staining. The photographs of the gels were taken with SONY Cyber-Shot, 10.1 mega pixels.

Data analysis

Differences in the digested PCR products were analyzed and scored on the basis of the presence or absence of a particular band that appeared on gel. The numerical taxonomy and multivariate analysis system NTSYS-PC software 2.01 (Rohlf, 2000) was used to compute Jacquard's coefficients of similarity. The observed data was used for the construction of a dendrogram with the help of unweighted pair group method with arithmetic mean (UPGMA) algorithm (Santosoa et al., 2005).

RESULTS AND DISCUSSION

DNA isolation and amplification of *rps*11 gene

Members of Apocynaceae are sometimes problematic when trying to obtain high quality DNA for PCR reactions, which is due to the existence of secondary metabolic products and/or latex in these species (Maliyakal, 1992). DNA was extracted using CTAB method and extracted DNA was checked on 1% agarose gel for determining the quality of DNA. The isolated DNA was used as a template for the amplification of *rps*11 gene and the amplification was confirmed by running the PCR amplified product on 1.5% agarose gel (Figure 1). The amplified product was subjected to restriction digestion with seven different restriction enzymes and was run on 12% PAGE.

Banding pattern exhibited by different restriction enzymes

There are many studies in plants mentioning chloroplast DNA (cpDNA) variation as a phylogenetic marker (Clegg and Zurawski, 1992), and the most widespread methodology involves restriction digestion followed by electrophoretic separation (Dowling et al., 1990). In CAPS technique, amplified product from genomic DNA can be obtained by using a pair of specific primers and the allele specific PCR amplified products are treated with different restriction enzymes. The variation in the length of the restricted products represents the presence or absence of a particular restriction site (Konieczny and Ausubel, 1993).

The CAPS markers usually separate in a co-dominant manner, which results in the discrimination of homozygous from heterozygous genotypes. Such type of marker system can be used in the genetic investigations

Table 2. Digestion pattern produced by *rps*11 gene amplified from seven species of Apocynaceae.

Species	Approximately digested fragment sizes (bp)							Total no. of bands
	***Scrf*I**	***Tsc*AI**	***Dpn*I**	***Hinf*I**	***Bsi*HKAI**	***Mse*I**	***Bse*GI**	
H. longifolia	250+170	290+130	320+100	310+110	320+100	280+140	250+170	14
W. volubilis	250+170	320+100	210*	250+170	320+100	280+140	250+170	13
T. cordata	250+170	290+130	210*	310+110	320+100	280+140	250+170	13
C. edulis	250 +170	290+130	210*	320+100	240+180	280+140	250+170	13
C. tuberculata	250+170	290+130	210*	320+100	240+180	280+140	250+170	13
T. hirsute	250+170	290+130	210*	320+100	320+100	280+140	250+170	13
C. buchananii	250+170	290+130	210*	320+100	300+120	280+140	250+170	13
Total no. of bands	14	14	8	14	14	14	14	92

*The amplified *rps*11 gene was equally divided in to two fragments with same molecular weight, therefore the appearance of such equal fragments were as a single thick band.

related to plant species (Ince et al., 2010).

Presently, seven different restriction enzymes (*Tsc*AI, *Scrf*I, *Dpn*I, *Bsi*HKAI, *Mse*I, *Hinf*I and *Bse*GI) were used for digesting the *rps*11 amplified product from seven different species. Seven restriction enzymes have produced 92 fragments with molecular weights ranging from ~100 bp to ~320 bp. Enzymes such as *Scrf*I, *Mse*I and *Bse*GI have shown monomorphic fragments in all taxa (Table 2), while other enzymes have shown variations among the samples producing different restricted fragments. Although, the restriction enzymes are highly specific for their cutting fashion but some factors such as methylation or mutation in restriction site can affect their efficacy (Vekemans et al., 1998). It was observed that *Tsc*AI enzyme has produced similar pattern in all species except *W. volubilis*, while *Dpn*I has shown variation in cutting pattern only in *H. longifolia* and has produced two bands of different molecular weights. High frequency in site variation was depicted by *Hinf*I revealing 100% polymorphism among the taxa and has produced 8 bands with small differences in molecular weights (Table 2).

Similarly, *Bsi*HKAI has produced different banding pattern in *C. edulis* and *C. buchananii*. It has already been reported that the polymorphism can be detected by CAPS technique and it is based on the sequence variations in the flanking regions. The change in nitrogenous bases (insertion/deletion) can be detected and it may help in more accurate identification of genotypes (Ince et al., 2010). Banding pattern has also shown partial digestion of the fragments, which lead to failure or the weak appearance of the bands on the gel (Culver and Noller, 1999).

UPGMA cluster analysis

UPGMA cluster analysis was carried out based on the restriction fragments produced by all the restriction enzymes among seven species belonging to different tribes of subfamilies Asclepiadoideae and Periplocoideae.

Three species namely: *H. longifolia*, *T. cordata*, and *W. volubilis* belong to tribe Marsdenieae, *T. hirsuta* is from Asclepiadeae, *Caralluma* species (*C. tuberculata* and *C. edulis*) are from Ceropegieae and one member of subfamily Periplocoideae *Cryptolepis buchananii* belongs to Cryptolepideae (http://www.bio.unibayreuth.de/planta2/research/databases/delta_as/index.htm). Cluster analysis revealed mixed pattern; *H. longifolia* and *T. cordata* appeared in a group at a significant genetic distance from *T. hirsuta* and *W. volubilis*, reflecting prolonged genetic isolation among the members of tribe Marsdenieae (Figure 2). Further, *Caralluma* species has shown 100% similarity level and both are closely related to *T. cordata* and *T. hirsuta*. Moreover, *T. hirsuta*, *C. buchananii* and *Caralluma* species appeared parallel to each other at 84% similarity coefficient. It was also observed that cluster has diverged at 58% similarity coefficient and *W. volubilis* has shown parallel lineage to large clade including other six species (Figure 2).

The results are an indicator of some changes at cpDNA level during the course of evolution and large genetic distances were revealed among the seven species of Apocynaceae.

Similarly, in a recent report, RAPD based data has shown a high level of genetic diversity between the two members of Ceropegieae namely, *C. tuberculata* and *C. edulis* (Mahmood et al., 2010) indicating that members of this family are genetically more diverse. However, additional molecular data from other regions of cpDNA is required to further resolve the genetic relationships among these species.

Conclusion

It has been observed from the data obtained that the

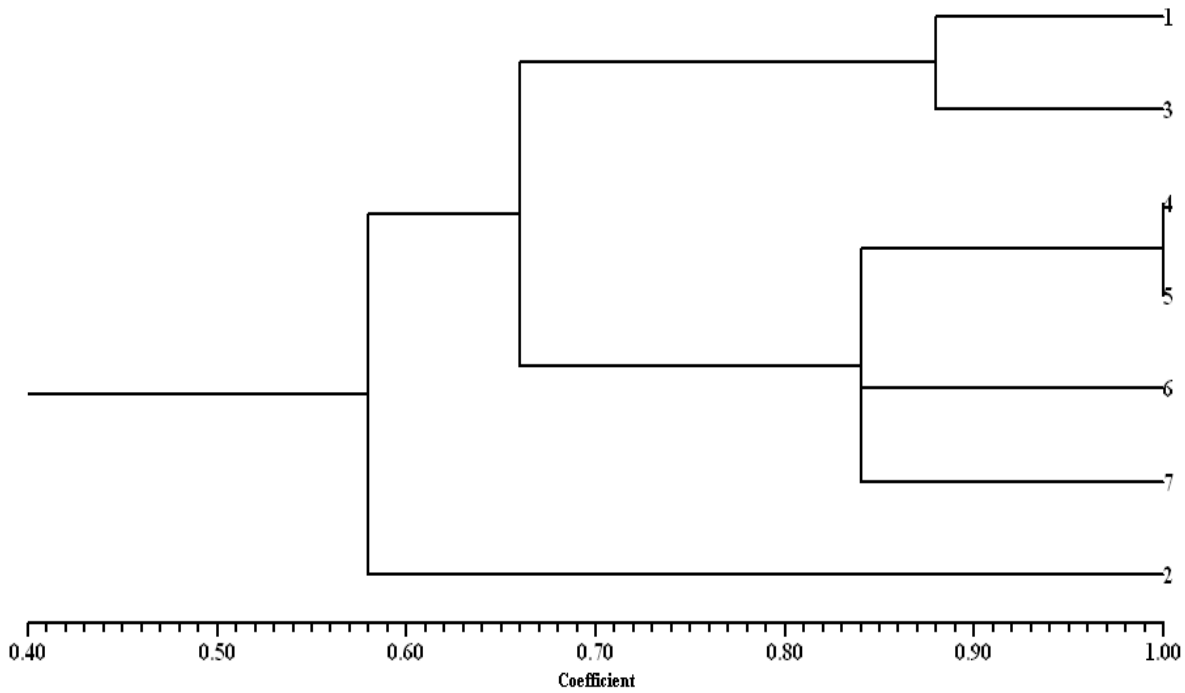

Figure 2. Phylogenetic relationship among seven Apocynaceae species based on restriction pattern of *rps*11 gene. 1: *Hoya longifolia*, 2: *Wattakaka volubilis*, 3: *Telosma cordata*, 4: *Caralluma edulis*, 5: *Caralluma tuberculata*, 6: *Tylophora hirsuta*, 7: *Cryptolepis buchananii*.

species get clustered in mixed pattern and closely related species appeared at higher genetic distances. Importantly, members of tribe Marsdeniea have shown a high level of genetic variation.

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