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Genetic diversity of selected Apocynaceae species based on chloroplast gene rps11

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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 (rps11). For this purpose, DNA was extracted from young leaves and with the help of a pair of primer, rps11 gene was amplified and seven restriction enzymes namely: TscAI, ScrI, DpnI, BsiKHAI, Msel, HinII, BseGI were used to digest the amplified rps11 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 rps11 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 bowl 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 bulb 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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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 (rps11). 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 8plus, Wealtech). The concentration of the DNA was measured by spectrophotometer (Smart SpectTM Plus) at 260 nm and high quality DNA was used for the amplification purposes.

**Primers designing and amplification of rps11 gene**

A pair of primer was designed from tobacco chloroplast genome (Accession # Z00044.2) available in Genbank for the amplification of rps11 gene, using the available online program Primer '3'. The sequence of the primers is as follows:

rps11 F: 5’ TGGCAAAAGCTATACCGAAAA 3’
rps11 R: 5’ TCCGAGGTCTACAGCCATT 3’

Genomic DNA was used as template for the amplification of rps11.

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

<table>
<thead>
<tr>
<th>S/N</th>
<th>Species name</th>
<th>Site name</th>
<th>Longitude and latitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hoya longifolia</td>
<td>Chakothi (Azad Kashmir)</td>
<td>73° 75'E and 33° 36'E</td>
</tr>
<tr>
<td>2</td>
<td>Wattakaka volubilis</td>
<td>Islamabad</td>
<td>33° 42'E and 73° 10'E</td>
</tr>
<tr>
<td>3</td>
<td>Telosma cordata</td>
<td>Chakothi (Azad Kashmir)</td>
<td>73° 75'E and 33° 36'E</td>
</tr>
<tr>
<td>4</td>
<td>Caralluma edulis</td>
<td>Manwali</td>
<td>32° 38'E and 71° 28'E</td>
</tr>
<tr>
<td>5</td>
<td>Caralluma tuberculata</td>
<td>Manwali</td>
<td>32° 38'E and 71° 28'E</td>
</tr>
<tr>
<td>6</td>
<td>Tylophora hirsuta</td>
<td>Islamabad</td>
<td>33° 42'E and 73° 10'E</td>
</tr>
<tr>
<td>7</td>
<td>Cryptolepis buchananii</td>
<td>Islamabad</td>
<td>33° 42'E and 73° 10'E</td>
</tr>
</tbody>
</table>
The conditions used for PCR amplification were pre PCR denaturation at 94°C for five minutes followed by 35 cycles of:

- Denaturation at 94°C for one minute.
- Annealing at 60°C for one minute.
- 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 MgCl₂ 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 rps11 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 rps11 gene from seven species of Apocynaceae. In total, seven restriction enzymes were chosen to digest the amplified rps11 gene. The names of the restriction enzymes used for digestion of the rps11 gene from selected species were TscAI, ScrI, Dpnl, BsiHKAI, Msel, HinfI and BseGI.

**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 rps11 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 rps11 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.
related to plant species (Ince et al., 2010). Presently, seven different restriction enzymes (TscAI, ScrI, DpnII, BseHKAI, MseI, HinII and BseGI) were used for digesting the rps11 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 ScrI, MseI and BseGI 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 TscAI enzyme has produced similar pattern in all species except W. volubilis, while DpnII 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 HinII revealing 100% polymorphism among the taxa and has produced 8 bands with small differences in molecular weights (Table 2).

Similarly, BseHKAI 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 Cryptoplepieae (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

### Table 2. Digestion pattern produced by rps11 gene amplified from seven species of Apocynaceae.

<table>
<thead>
<tr>
<th>Species</th>
<th>Approximately digested fragment sizes (bp)</th>
<th>Total no. of bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. longifolia</td>
<td>250+170 290+130 320+100 310+110 320+100 280+140 250+170</td>
<td>14</td>
</tr>
<tr>
<td>W. volubilis</td>
<td>250+170 320+100 210* 250+170 320+100 280+140 250+170</td>
<td>13</td>
</tr>
<tr>
<td>T. cordata</td>
<td>250+170 290+130 320+100 310+110 320+100 280+140 250+170</td>
<td>13</td>
</tr>
<tr>
<td>C. edulis</td>
<td>250+170 290+130 320+100 310+110 320+100 280+140 250+170</td>
<td>13</td>
</tr>
<tr>
<td>C. tuberculata</td>
<td>250+170 290+130 320+100 310+110 320+100 280+140 250+170</td>
<td>13</td>
</tr>
<tr>
<td>T. hirsuta</td>
<td>250+170 290+130 320+100 310+110 320+100 280+140 250+170</td>
<td>13</td>
</tr>
<tr>
<td>C. buchananii</td>
<td>250+170 290+130 320+100 300+120 280+140 250+170</td>
<td>13</td>
</tr>
<tr>
<td>Total no. of bands</td>
<td>14 14 8 14 14 14 14</td>
<td>92</td>
</tr>
</tbody>
</table>

*The amplified rps11 gene was equally divided into two fragments with same molecular weight, therefore the appearance of such equal fragments was as a single thick band.*
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.

REFERENCES


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