Arch. Biol. Sci., Belgrade, 63 (3), 661-665, 2011 DOI:10.2298/ABS1103661M

PHYLOGENETIC ANALYSIS OF DIFFERENT *ARTEMISIA* SPECIES BASED ON CHLOROPLAST GENE *RPS*11

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Abstract - *Artemisia* L. of the family Astereacae is a genus with enormous economical and medicinal importance. As a result, the genus *Artemisia* has been the subject of diversity-focused studies. In the present study, phylogenetic analysis based on restriction fragment length polymorphism of the chloroplast *rps*11 gene was conducted on eight species of *Artemisia* that represent the major morphological subgroups. After digestion of the *rps*11 gene that was amplified from eight species, with six different restriction enzymes, each restriction site was observed and scored on a 12% polyacrylamide gel. The data were analyzed using the Numerical Taxonomy and Multivariate Analysis System to infer the phylogenetic relationship within the genus. A mixed pattern was observed among the species belonging to various taxonomic groups of *Artemisia*.

Key words: Artemisia, chloroplast gene, CAPS, phylogenetic analysis

UDC 582.998.16:581.174:575

INTRODUCTION

Artemisia is considered to be the largest genus present in the tribe Anthemideae and it is the largest genera in the family Asteraceae, having more than 500 taxa (the number varies depending on different reports: McArthur, 1978; Ling, 1982; Mabberley, 1990; Bremer and Humphires, 1993; Oberprieler, 2001; Valles and McArthur, 2001). *Artemisia* is widely distributed in the northern hemisphere with two of its main speciation centers present in western and central Asia and a few of its representatives present in the southern hemisphere. Pakistan is considered to be one of the centers of origin of *Artemisia* species and many species of *Artemisia* have been reported (Pareto, 1985; Tan et al., 1998). After different systematic reshufflings, the genus was divided into five large groups; *Absinthium* DC., *Artemisia* L., *Dracunculus* Besser, *Seriphidium* Besser and *Tridantatae* (Rydb.) (Torrell et al., 1999).

The genus *Artemisia* is an important medicinal plant and also has high economical value with respect to its usage as food, forage and ornamental plants (Barney and DiTommase, 2003). It has also been reported that in many countries of the Middle East and Turkey many species of *Artemisia* are used as herbal medicines for the treatment of diabetes, high blood pressure and gastrointestinal ailments (Mossa, 1985; Al-Shamaony et al., 1994; Subramoniam et al., 1996). *Artemisia* species such as *A. annua* and *A. indica* contain a drug called *artemisinin* that has been used for treating malaria in China and Thailand for over 2000 years (Bunyapraphatsara, 1986; Farnsworth, 1992; Ashraf et al., 2010). *A. dubia* has been used in Magar of Bukini, Baglung, Western Nepal as a stomachic and purgative, and for asthma and skin diseases such as scabies and ulcers (Sapkota, 2008). *A. absinthium* L. is known to possess ethno-medical and biological properties related to anthelmintic (Tariq et al., 2009), antifungal (Kordali et al., 2005) and antimicrobial activities

(Lopes-Lutz et al., 2008). Moreover, Afsanteen (*A. brevifolia*) is widely used in the ethno-veterinary medicine system of Pakistan as an anthelmintic plant (Iqbal et al., 2004).

Different studies have been conducted to measure the genetic diversity or phylogenetic analysis of *Artemisia* species by using different molecular markers (Mohsen and Ali, 2008; Hayat et al., 2009; Nazar and Mahmood, 2010). Besides the other molecular markers related to techniques for phylogenetic studies, cpDNA is a genome frequently used in population genetic studies (Comes and Abbott, 1999), and it has successfully been used for phylogenetic analysis (Ziegenhagen and Fladung, 1997). Polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) has the ability to discriminate between genotypes based upon the presence or absence of restriction sites within the amplified DNA. This technique is also called Cleaved Amplified Polymorphic Sequence (CAPS) (Karp et al., 1997). In the present study, the phylogenetic relationship amongst eight *Artemisia* species has been carried out with the help of a PCR-RFLP-based analysis of *rps* 11, a gene encoding chloroplast ribosomal protein 11.

MATERIALS AND METHODS

Collection of plant material

Artemisia samples were collected from different areas of Pakistan (Table 1).

Primer designing and amplification of the rps11 gene

A pair of primers was designed from tobacco chloroplast genome (Accession # Z00044.2) available in Genbank for the amplification of ribosomal protein of smaller subunit 11 (*rps* 11). Primers were designed with the help of online program Primer 3. The sequence of primers is as follows;

*rps*11F: 5’ TGGCAAAAGCTATACCGAAAA 3’

*rps*11R: 5’ TTCGGAGGTCTACAGCCATT 3’

Table 1: List of *Artemisia* species collected from different regions of Pakistan.

Sr.No.	Species name	Location
1	A. brevifolia	Kaghan
2	A. japonica	Rawalakot (AJK)
3	A. vulgaris	Rawalakot (AJK)
4	A. tangutica	Naran
5	A. tournifortiana	Ayubia (Pipeline track)
6	A. roxburghiana	Rawalakot (AJK)
7	A. dubia	Ayubia (Pipeline track)
8	A. persica	Kaghan

The PCR conditions used for amplification of the *rps*11 gene were pre-PCR denaturation at 94˚C for 5 min followed by 35 cycles of denaturation at 94˚C for 30 s, annealing at 55˚C for 30 s and extension at 72˚C for 1 min. Final extension was carried out at 72˚C for 20 min and the PCR reaction contents were held at 4˚C till further use. About 25 µl of the PCR mixture contained, 12.5 µl of PCR Master Mix (Fermentas), 9.5 µl PCR water, 1 µl of each primer (25 pM) and 1 µl of DNA template was used.

Cleaved Amplified Polymorphic Sequence (CAPS)

Restriction enzymes were selected by digesting the already available sequence of rps11 gene using the online tool NEB cutter (http://tools.neb.com/NEBcutter). Then, the selected restriction enzymes were used for the digestion of the rps11 gene amplified from eight *Artemisia* species. The digestion mixture (containing the PCR amplified product (10µl), nuclease free water (7 µl), 10X Buffer (2µl), and enzyme (5 units; 1µl) (Fermentas)) was incubated overnight. After incubation, the digested product was subjected to 12% polyacrylamide gel (BioRad) electrophoresis and then silver stained. The photographs of the gels were taken with a SONY Cyber-Shot 10.1 mega pixel camera.

DATA SCORING AND ANALYSIS

The presence of a particular restricted fragment was marked as “1” and its absence as “0”. For phyloge-

Table 2: Total number of restricted fragments produced by different restriction enzymes among eight species of *Artemisia*.

S.No.	Species	*Scrf* I	*Tsc*AI	*Dpn*I	*Hinf* I	*BsiHKAI*	*FokI*
1	A. brevifolia	2	2	2	2	1	1
2	A. japonica	2	2	2	2	1	1
3	A. vulgaris	1	2	2	1	2	1
4	A. tangutica	2	1	2	2	1	1
5	A. tournifortiana	2	2	2	2	1	2
6	A. roxburghiana	2	1	2	1	1	1
7	A. dubia	2	2	2	1	1	2
8	A. persica	2	2	2	2	1	1
	Total	15	14	16	13	9	10
	Percentage	19.4%	18.18%	20.7%	16.8%	11.6%	12.9%

netic analysis, the Numerical Taxonomy and Multivariate Analysis System (NTSYs) PC software 2.01 (Rohlf, 2005) was used to construct a dendrogram by Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

RESULTS AND DISCUSSION

After various taxonomic studies the genus *Artemisia* was divided into five large groups; *Absinthium* DC., *Artemisia* (=*Abrotanum* Besser), *Dracunculus* Besser, *Seriphidium* Besser and *Tridantatae* (Rydb.) (Torrell et al., 1999). However, this infrageneric classification does not represent natural groups and is not accepted by all taxonomists (Persson, 1974; McArthur et al., 1981; Valles and McArthur, 2001; Valles and Garnatje, 2005). Through various molecular studies (Sanz et al., 2008; Tkach et al., 2007; Valles et al., 2003; Watson et al., 2002) it was concluded that the genus may need more molecular evidence to resolve the confusions at the infrageneric level. In the present phylogenetic analysis an attempt has been made to find out the relationships among different species of genus *Artemisia* by using the PCR-RFLP technique.

Eight *Artemisia* species were collected from different parts of Pakistan and the plants were preserved at -20˚C. High quality DNA was isolated from the leaves of preserved plants by the CTAB (cetyl trimethyl ammonium bromide) isolation procedure (Richard, 1997). The isolated DNA was used for amplification of the *rps*11 gene. The amplified product of approximately 400 bp was subjected to restriction digestion with six different restriction enzymes (*Scrf***I,** *Tsc***AI,** *Dpn***I,** *Hinf***I,** *BsiHKA*I and *Fok*I). The digested product was run on 12% PAGE. In total, 77 restriction fragments were observed: the highest number of fragments was produced by *Dpn*I (16 fragments), and the lowest by *BsiHk*AI (9 fragments). It was also observed that the amplified *rps*11 gene remained un-

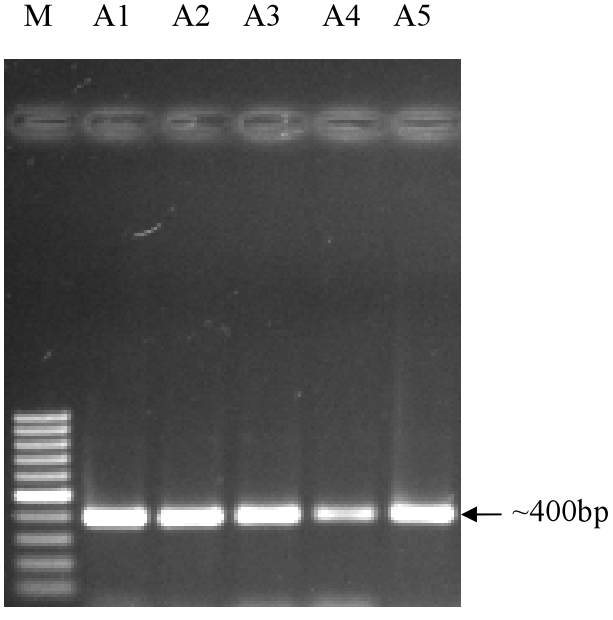

Fig. 1: Amplification of *rps*11 gene from different species of *Artemisia*; M: Ladder 100 bp, 1 to 5 - amplified fragments from different *Artemisia* species.

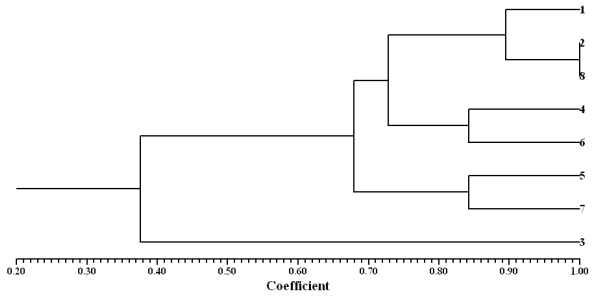

Fig. 2: Dendrogram showing the phylogenetic relationships amongst eight *Artemisia* species based on digestion pattern of *rps*11 gene. 1 - *A. brevifolia*; 2 - *A. japonica*; 3 - *A. vulgaris*; 4 - *A. tangutica*; 5 - *A. tournifortiana*; 6 - *A. roxburghiana*; 7 - *A. dubia*; 8 - *A. persica.*

digested in some species probably due to site variations (Table 2).

Phylogenetic analysis

The data obtained from the RFLP of the *rps*11 gene was used to reveal the phylogenetic relationship among the species of *Artemisia*. UPGMA cluster diverge at 38% similarity coefficient into two lines. All the species grouped into one cluster, except *A. vulgaris* which was parallel to all the other species. Earlier, Barney and DiTommase (2003) reported a high level of intraspecific diversity in *A. vulgaris* in comparison to other species of *Artemisia*. Moreover, *A. japonica* and *A. persica* of sections *Dracunculus* Besser and *Absinthium* DC, respectively, appeared at 100% similarity, thus revealing a very close association during the evolutionary process. Furthermore, both species showed a close link with *A. brevifolia* (belonging to section *Tridantatae* (Rydb.) McArthur). It was observed that these three species formed a group and they showed a relationship with *A. tangutica* and *A. roxburghiana* at 74% similarity coefficient. Similarly, *A. tournifortiana* and *A. dubia* diverged at 68% similarity coefficient.

It is evident from the data that mixing had occurred at an infrageneric level during the evolutionary process. Sometimes geological and ecological factors also affect the genetic characterization and organization of diversity (Loveless and Hamrick, 1984). To resolve the problem of genus *Artemisia* at an infrageneric level there is a need to study other genomic regions to produce groups depicting natural classification.

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