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*Full Length Research Paper*

# Phylogenetics of selected *Plantago* species on the basis of *rps*14 chloroplast gene

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***Plantago,* principal genus of Plantaginaceae family, constitutes about 260 species which are considered medicinally important due to a wide range of biological activities that have been found from the plant extracts. Since ancient times *Plantago* species have been extensively used for treating diversified diseases and their use is still prevalent today in both traditional and in modern medicine. For insight in genetic makeup of the *Plantago* species polymerase chain reaction-restricted fragments length polymorphism (PCR-RFLP) was used in this study for analysis of similarities and differences within and among species. PCR-RFLP of *rps*14 gene in three *Plantago* species (*Plantago ovata, Plantago lanceolata* and *Plantago maxicana*) showed high degree of similarity and low genetic diversification among the selected species. This study concluded on that the *rps*14 gene sequences are highly conserved among the selected species and only about 14% polymorphism was observed.**

**Key words:** Phylogeny, chloroplast DNA, plantago, polymerase chain reaction-restricted fragments length polymorphism (PCR-RFLP).

## INTRODUCTION

The *Plantago* plant constitutes the principal genus of the *Plantaginaceae* family. About 260 *Plantago* species have been found in temperate regions and in tropical zones (Van der Aart and Vulto, 1992), including varied ecological systems required by the plant to adapt both phenotypically and physiologically (Kuiper, 1992; Van Delden et al., 1992). Among genus *Plantago*, only two species namely *Plantago ovata* and *Plantago psyllium* have been extensively used for the production of seed, seed husk and mucilage (active substance) which are used in pharmacy and other industries such as pulp and paper production, loom, military and petroleum extraction (Chevallier, 1996; Mohebbi, 2000). A wide range of biological activities has been found from *Plantago* species, plant extracts or isolated compounds including treating diseases related to skin, wound healing, inflammation, disorders of respiratory and digestive organs, reproductive system, blood circulation and cancer (Samuelsen, 2000). With the advancements of molecular techniques particularly the development of molecular markers is playing a an important role in revealing DNA sequence similarities and differences within and among species. Molecular markers are of different types such as morphological, biochemical and DNA based molecular markers. Molecular markers are basically of two types, of polymerase chain reaction (PCR) based molecular markers and non-PCR based markers. PCR based markers include amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), simple sequence repeats (SSR), cleaved amplified polymorphic sequence (CAPS) etc. (Kumar et al., 2009). Biometric and molecular techniques have been used to assess the genetic variability and relatedness of 80 germplasm accessions of *Plantago* species (*P. ovata, P. lanceolata, Plantago major*). Among these 80 accessions, thirty six accessions were analyzed through RAPD profiling for similarity and genetic distances using 20 random primers. It was observed that Intraspecific differences in all three species are smaller than interspecific diversity. These highly divergent lines could be used to produce superior hybrids (Singh et al., 2009). In the present study phylogenetic relationship among three selected *Plantago* species namely *P.* ovata,

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*P. lanceolata* and *P. maxicana* was studied using polymerase chain reaction-restricted fragments length polymorphism (PCR-RFLP) (CAPS) technique which is one of the rapid and reliable marker techniques for determining genetic relationship and for DNA typing.

## MATERIALS AND METHODS

### Plant material

Three *Plantago* species namely *P. ovata*, *P. lanceolata* and *P. maxicana* were collected from murree Pakistan. Young leaves were removed from plants and stored at 4°C in plastic bags.

### DNA extraction

Total genomic DNA was extracted from plant material by using Cetyl Trimethyl Amonium Bromide (CTAB) method (Richards, 1997) with few modifications. Approximately 0.3 g of plant material (about two to three leaves) was crushed with the help of chilled pestle and mortar, by adding 1 ml of preheated (65°C) 2 × CTAB buffer. The homogenized leaf tissues were transferred to 1.5 ml eppendorf tubes and incubated at 65°C for 45 min. The tubes were then centrifuged for 10 min at 10,000 rpm and supernatant was collected and transferred to new eppendorf tubes. An equal volume of chloroform-isoamylalcohol (24:1) was added to the supernatant then after inverting tubes five to six times eppendorf tubes were centrifuge at 10,000 rpm for 10 min. This step was repeated three to four times then an equal volume of chilled isopropanol was added to the supernatant and eppendorf tubes were inverted gently for two to three times and placed at -20°C for 20 to 30 min and after that centrifugation was done at 10,000 rpm for 10 min. The white DNA pellet was precipitated out and stick to the base of eppendorf tubes again, the supernatant was discarded and then ethanol washing was performed with 70% cold ethanol. After discarding ethanol the DNA pellet was air dried at room temperature and then resuspended in a mixture of 30 to 40 µl TE (Tris EDTA) buffer containing 10 mg/ml of RNase. The DNA samples were then incubated at 37°C for 30 min and purified samples were stored at -20°C for further use. DNA presence in the samples and its quality was assessed by running DNA sample mixed with loading dye (Bromophenol blue) on 1% agrose gel in 0.5X TAE (Tris-Acetate-EDTA) buffer.

### Designing primer

A pair of primer that amplify ribosomal protein S14 (*rps*14) were designed from tobacco chloroplast genome (Accession # Z00044.2) present in NIH (National Institute of Health, United States of America) genetic sequence database, GenBank. Primer were designed by using online available Primer 3 (version 0.4.0) software (http//primer3.source-forge.net/). The primer sequences are as follow:

*rps*14F *forward primer* 5′
ATGGCAAGGAAAAGTTTGATTC 3′
*rps*14R *reverse primer* 5′
TTACCAACTTGATCTTGTTGCTCCT 3′

### Polymerase chain reaction

For the amplification of *rps*14 gene sequences the PCR conditions that were used included pre PCR denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 60°C for *P. ovata* and *P. maxicana*, annealing for 1 min at 57°C for *P. lanceolata* and extension at 72°C for 1 min. Final cycle was same except extension at 72°C for 6 min. All the conditions were same for three species except for annealing temperature.

About 25 µl of PCR mixture contained 12.5 µl of 2× PCR Master Mix (Fermentas), 9.5 µl of nuclease free water, 50 pmol (1 µl) of forward and reverse primer each and 1 µl of DNA template was used.

To check the successful amplification of the desired gene and to analyze the PCR product quality, a mixture of PCR product and loading dye was run on 1.5% agrose gel in 0.5X TAE (Tris-Acetate-EDTA) buffer.

### Restriction enzyme mapping

Restriction enzymes were mapped on the available sequences of *rps*14 gene from tobacco chloroplast genome (Accession # Z00044.2) using the online tools such as NEBcutter, version 2.0 (http//tools.neb.com/NEBcutter) and Restriction Mapper (version 3.0). The restriction data obtained was applied on the amplified *rps*14 gene from three species of *Plantago*.

### Restriction digestion of PCR product

Amplified PCR products were restricted by using 6 different restriction enzymes. Digestion was performed by using digestion mixture containing 3µl of nuclease free water, 1.5 µl of 10X Buffer, 10 µl PCR product and 0.5 µl of restriction enzyme. The digestion mixture was short spin (1000 rpm for 30 s) and then incubated at recommended (Fermentas) temperature for 16 h. The restriction enzymes used for cleaving *rps*14 gene are *Alw*21I (*Bsi*HKAI), *Bme*1390I (*Scr*FI), *Bsu*RI (*Hae*III), *Eam*1104I (*Ear*I), *Alw*44I (*Apa*LI) and *BspP*I (*Alw*I).

### Polyacrylamide gel electrophoresis (PAGE)

To check the digestion pattern in restricted samples PAGE was done. The digested samples were mixed with loading dye (Bromophenol blue) and then loaded in wells. Samples were run on 16% PAGE (Mini-PROTEAN® Tetra Cell, Bio-Rad), a 50 bp ladder (Fermentas) was also loaded in one well to estimate the molecular sizes of digested fragments. The gel was run at 80 V for approximately 3 h and then ethidium bromide staining was performed.

Finally, the gel was visualized under UV light and gel documentation was carried out by Dolphin Doc $^{Plus}$ gel documentation system (Wealtec).

### Data scoring and analysis

Banding patterns from ethidium bromide stained polyacrylamide gels were used for data analysis. The presence of a particular band was scored as “1” and absence as “0”. The positions of restricted fragments were compared with 50 bp ladder (Fermentas). Phylogenetic analysis was carried out using Numerical Taxonomy and Multivariate Analysis System (NTSys) PC software 2.01 (Rohlf, 2000) to compute the Jacquard’s coefficient of similarity and dendrograms were constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Santosoa et al., 2005).

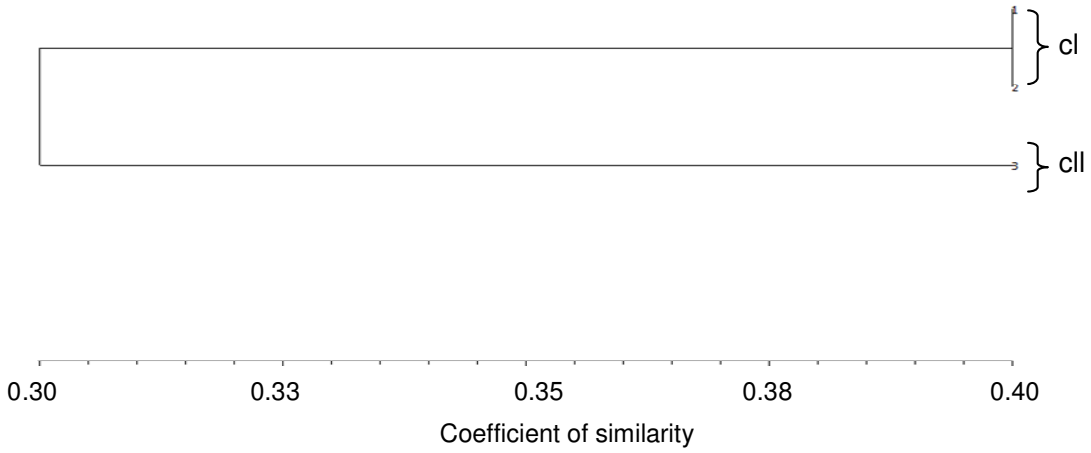


**Figure 1.** Dendogram showing the phylogenetic relationship among three *Plantago* species based on *rps*14 gene cleaved by *Bsp*PI restriction enzyme.

## RESULTS

### DNA extraction and polymerase chain reaction amplification

Successful DNA extraction and amplification *rps*14 gene was achieved from all three species. The product size of amplified product was approximately 320 bp. Annealing temperature was same for two species that is *P. ovata* and *P. maxicana* while amplification was optimized at slightly lower annealing temperature for *P. lanceolata*.

### Banding pattern produced by *Alw*21I, *Alw*44I and *Bme*1390I

Total numbers of bands observed were six upon cleavage by *Alw*21I, *Alw*44I and *Bme*1390I restriction enzymes. Two sets of monomorphic bands were produced by these three enzymes. In case of *Alw*21I and *Alw*44I, one set of bands produced was of approximately 170 bp and the other set was of approximately 150 bp. While one set of bands produced was of approximately 250 bp and the other set was of approximately 70 bp in case of *Bme*1390I restriction enzyme. Overall, on the basis of these three enzymes In case of these three enzymes it was observed that there is 100% similarity among studied *Plantago* species.

### Banding pattern observed upon cleavage by *Bsu*RI and *Eam*1104I

*rps*14 amplified region of *Plantago* chloroplast genome produced only single band for all species after digesting with *Bsu*RI and *Eam*1104I restriction enzymes. The band size produced is approximately 320 bp which is exactly the same as that of the amplified product, showing that no digestion was carried out by these this showed that no digestion took place by these particular enzymes and thus an indication of the absence of cleavage site for *Bsu*RI and *Eam*1104I restriction enzymes in *rps*14 gene sequence. Total numbers of bands on gel were three with equal molecular weight and all were monomorphic. So there was 100% similarity among *Plantago* species for these enzymes.

### Banding pattern produced upon digestion by *Bsp*PI

Total numbers of bands produced were 5 upon cleavage by *Bsp*PI restriction enzyme and polymorphic bands were observed during digestion. One set of band was produced in case of *P. ovata*, in which one band produced was of approximately 280 bp and the other was of approximately 40 bp. However, in the other set of band which was produced in *P. maxicana,* one band produced was of approximately 300 bp and the second one was of approximately 20 bp. There was no restriction observed in *rps14* gene amplified from *P. lanceolata*. On the basis of data observed it was revealed that there is polymorphism among studied *Plantago* species based on *Bsp*PI restriction site.

### Cluster analysis of *Bsp*PI restricted PCR product

The dendogram generated by UPGMA has two clusters. Cluster cI has 2 species: *P. ovata* and *P. maxicana*. The members of cI have shown 100% similarity coefficient with each other where as cluster cII only has (*P. lanceolata*) that act as an out group and showed 30% similarity with cluster cI (Figure 1). So it can be concluded that PCR-RFLP of *rps*14 gene by using *Bsp*PI enzyme

can be used as a specific marker for *P. lanceolata* identification and differentiation.

## DISCUSSION

In this study genetic diversity among three selected *Plantago* species was evaluated by using PCR-RFLP method on cpDNA sequences. Earlier work of Small et al. (2005) revealed that cpDNA sequences are the primary source of characters for phylogenetic studies in plants. *rps*14 region of *P. ovata*, *P. lanceolata* and *P. maxicana* was successfully amplified by using a set of primer (forward and reverse) that was designed from cpDNA sequence of *Nicotiana tabacum.* The size of *rps*14 gene in tobacco chloroplast genome is 303 bp, which may vary from plant to plant and family to family as in case of *Plantago* the size was observed to be approximately 320 bp for the selected species. In the present study, high monomorphism was observed among all three *Plantago* species for *Alw21*I, *Alw44*I, *Eam1104*I, *BsuR*I and *Bme*1390I enzymes. However, when *rps*14 fragment was treated with *BspP*I enzyme some polymorphism was observed. Within the genus the level of polymorphism detected was very low, this might be due to self pollinated nature of plant or could be because of restricted distribution, non-effective gene flow, low fecundity, local selection pressure, low pollen flow, inbreeding systems or less possibility of introgressions during evolution (Loveless and Hamrick, 1984; Loveless, 1992). Different molecular markers have been used including the widely used RAPD markers (Mahmood et al., 2010a, b; Mahmood et al., 2011a, b, Nazar and Mahmood, 2011; Shinwari et al., 2011;), AFLP markers (Shaheen et al., 2010) for studying the genetic diversity, but the use of PCR-RFLP is now in routine for such diversity related studies mainly due to the low template DNA requirements and high reproducibility. This technique also has another plus point that does not require Southern blot hybridization and radioactive detection. According to Kumar et al. (2009) PCR-RFLP tells about restriction site sequence variation in terms of restricted fragments length polymorphism.

### Conclusion

Results of phylogenetic analysis indicated that genus *Plantago* showed high similarity and low genetic diversification among three selected *Plantago* species. Phylogenetic analysis based on PCR-RFLP of *rps*14 gene showed divergence of *P. maxicana* and *P. ovata* from *P. lanceolata* only in case of *Bsp*PI restriction enzyme. The polymorphism observed is only 14%. So, it can be concluded that *rps*14 gene sequences are highly conserved in genus *Plantago* and it does not provide much information for establishing phylogeny of genus *Plantago*. High similarity among species also indicated that these species are monophyletic.

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