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Investigation of Genetic Diversity among *Medicago* species Using RAPD Markers

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Authors' contributions

This work was carried out in collaboration between all authors. Authors KM and KM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MA, AK, and NA managed the analyses of the study. Authors MSN and WA managed the literature searches. Authors HA and ZA contributed in final manuscript writing. All authors read and approved the final manuscript.

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ABSTRACT

Aims: *Medicago* is known as the Queen of forage with potential economic importance to our society. The present study aimed at the use of RAPD-PCR DNA marker to identify the genetic fingerprints affinities of six species of Alfalfa.
Place and Duration of Study: The study was conducted at the Department of Genetics, Garden Campus, Hazara University, Mansehra Pakistan during February, 2011 to August, 2013.
Methodology: In this study, six species of *Medicago* namely *TWAL* (Tetraploid Wisconsin Alfalfa Line), *Medicago arborea, Medicago falcata*, *Medicago sativa*, *Medicago lupulina* and *Medicago polymorpha* were used to explore the diversity of alfalfa. Seven out of 120 decamers produced 34 polymorphic loci with 100% polymorphism to identify the different species of *Medicago* crop. The

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range of polymorphic loci was observed from 300 to 700 bp. Eleven species specific loci were generated by seven decamers. Primer B-18 generated single specific locus 700 bp against genomic DNA of *M. lupulina* and it is important to identify particular species of Alfalfa. The bivariate data were recorded as the presence of locus 1 and absence 0 and then this data was transferred into A and C respectively to make it suitable for DNAMAN software (version 5.2.2.0; Applied Biostatistics Inc). Moreover, cluster analysis was performed using sequence alignment and divergence function of the DNAMAN against the bivariate data collected from the products of decamers. All members clustered in a unique pattern except *M. falcata* and *M. lupulina* those shared 86% homology. Three distinct groups were observed during UPGMA (Unweighted pair Group Method with Arithmetic Mean). During the phylogenetic study, TWAL was observed to have genetic diversity from other five species of Alfalfa.

Conclusion: So, the present study is enabling us to discriminate different species of Alfalfa and it could be useful to identify and authenticate different species of the same genus of medicinal important plant from the Flora of Pakistan.

Keywords: Medicago; RAPD; Genetic diversity.

1. INTRODUCTION

Plants are essential constituents of ecosystems. Among plants, *Medicago* is one of the important legume plants that have been widely studied because it has numerous agriculturally important and domesticated species and known as queen of forage [1-2]. There are 87 species that have been recorded and these are distributed mainly around the Mediterranean basin [3-4]. A dozen annual species of *Medicago* have become significant pasture crops only in the past century, while best known species of this genus is *M. sativa* (alfalfa). It is the fourth most important crop in North America (after corn, soybean and wheat, respectively), and the temperate world's most important forage crop [5]. Several modern technologies are now being developed that have huge promise for extending the importance of alfalfa and its allied species well beyond their present primary use as a feed for livestock [5].

There are many effective and sensitive techniques which are being extensively used for characterization and conservation of crops and valuable plants [6-8]. Molecular marker can be employed for analysis of variance at the DNA level and different markers with useful genetic qualities (they can be dominant or co-dominant, can amplify anonymous or characterized loci, can contain expressed or non-expressed sequences, etc.) are being used by many researchers [9-12]. Analyses of genetic diversity are usually based on assessing the diversity of molecular markers, which tend to be selectively neutral [13].

Recently, many researchers have used the RAPD technique to estimate genetic diversity of various endangered plant species and valuable crops [14-18]. Random Amplified Polymorphic DNA (RAPD) is now in common use for ecological, evolutionary, taxonomy, phylogeny and genetic studies of plant sciences. These techniques are well established and their advantages and limitations have been documented properly [19].

In the present study, we attempted to discriminate, characterize and explore the genetics of valuable plants from Pakistan based on DNA markers and to increase the efficiency for study of crops at DNA level. It is the first comprehensive study at the DNA level of *Medicago* species from Pakistan using PCR based DNA markers. Our objective of the research was to identify specific regions of DNA linked to different species and establish clusters of species based on simple RAPD-PCR technique.

2. MATERIALS AND METHODS

2.1 Plant Material

Six species of *Medicago* namely *TWAL* (Tetraploid Wisconsin Alfalfa Line), *Medicago arborea, Medicago falcata*, *Medicago sativa*, *Medicago lupulina* and *Medicago polymorpha* were selected for the present study. Seeds of 3 species of *Medicago* were kindly provided by Prof. Habib Ahmad, Chairman, Department of Genetics, Hazara University, Mansehra. Hundred seeds of *TWAL* (Tetraploid Wisconsin Alfalfa Line), thirty-eight seeds of *Marborea* and hundred seeds of *M falcata* were planted in pots under greenhouse conditions at Garden Campus of Hazara University, Mansehra (Latitude

34°20'N, Longitude 7°15'E, Altitude 1066 meter) and leaves of 3 local varieties were also collected from the Garden Campus of Hazara University. For molecular study, one sample of each species was used to extract whole genomic DNA.

2.2 DNA Isolation

DNA was isolated from the leaves of *Medicago* species and two protocols were modified and optimized for DNA isolation from *Medicago,* Cetyl trimethyl ammonium bromide (CTAB) procedure [20] and a small scale DNA isolation procedure [21]. The quality and the quantity of DNA were estimated by capering with Fementas DNA molecular weight marker (cat. SM0403). The DNA was diluted to different concentrations (10 ng, 30 ng, 50 ng and 100 ng) in sterile double-distilled water for RAPD-PCR optimization reactions.

2.3 RAPD-PCR Primers

In the present study, RAPD primers were used against the genomic DNA of *Medicago* species to find genetic diversity. The primers used to identify the DNA marker linked to *Medicago* species were obtained from BIONEER.

2.4 RAPD PCR Optimization and Amplification of DNA Fragment

PCR mixture was prepared in sterile 200 µl tubes using commercially available kits (Wizbiosolutions, Korea), each mixture containing 50 ng of genomic DNA, 1x PCR buffer (75 mM Tris HCl PH 8.8 at 25ºC, 20 mM $(NH4)_2SO_4$ and 0.01 (Tween-200), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM deoxy nucleotide triphosphate (dNTPs; dATP, dCTP, dGTP, and dTTP, 10 pmol decamer arbitrary primers. The total volume of each reaction mixture was adjusted to 20 µl with sterilized water.

The amplification was carried out on DNA thermo cycler (Applied Biosystem., 2720 Thermocycler) with a modified version of the conditions used by William et al. [22]. It was programmed as single denaturation step for 5 min at 94ºC followed by a step cycle programmed for 40 cycles of denaturation at 94ºC for 1 min, annealing at 34 - 37ºC (depending upon best amplification based on GC content of decamers) for 1 min and extension at 72ºC for 2 min and final extension at 72ºC for 10 min.

2.5 Resolving of PCR Product

All PCR products were resolved by electrophoresis along with the DNA marker on 1.5% agarose gels, prepared in 1X TAE (Tris/Acetate/EDTA) buffer**.** The electrophoretic file images were documented and saved using Uvitech gel documentation (gel documentation system).

2.6 Scoring and Processing of RAPD Data

The amplification of PCR products was done twice or thrice for reproducibility of band scoring. The size of amplified RAPD-DNA fragments (bp) was estimated by reference to a known DNA marker. Only reproducible PCR products were scored and used in subsequent analyses. The banding pattern of RAPD-PCR was scored for the presence (1) or absence (0) of specific amplicon and then 1 transferred into A and 0 into C as DNAMAN software can read data in the sequence form (Table 1). Data were analyzed using multiple sequence alignment and divergence function of DNAMAN statistical software (version 5.2.2.0; Applied Biostatistics Inc). Based on the divergence matrix and using the neighbor-joining method (NJ), we constructed a dendogram showing the difference between six as determined with 7 primers from 22 binary characters in the amplicon presence – absence [23]. The Nei's [24] genetic distance based on Unweighted pair Group Method with Arithmetic Mean (UPGMA).

3. RESULTS AND DISCUSSION

Medicago is considered as one the most important genera of forage plants [25] and also used as medicine, human food, green manure sources of industrial enzymes in biotechnology [26], model genomic species [27], and model systems for the study of nitrogen fixation [28], Six species of *Medicago* (*Fabaceae*) were used for DNA analysis. Total genomic DNA from six genetic stocks of polyploid *Medicago* was isolated using small scale DNA isolation procedure [20-21,29].

3.1 Amplification of RAPD Primers

The highly purified genomic DNA samples from six selected species were subjected to analysis and characterization of genomic synteny among them with the help of RAPD-PCR. One hundred and twenty RAPD markers were obtained from BioNeer kits and applied against the six DNA samples. After initial screening, 7 RAPD primers

were chosen out of 120 primers for further study (Table 2). The selected 7 RAPD primers generated 34 scorable amplification products against six genomic DNA samples of *Medicago*. The results were analyzed by using DNAMAN software to generate homology and phylogenetic tree and various other parameters, i.e., total bands (TB), polymorphic bands (PB), monomorphic bands (MB), percentage of polymorphic (PP), were also considered.

3.2 Polymorphism and Polymorphic Content of Decamers

Polymorphism is the most important property of DNA marker, which can be used to discriminate the genetic basis of traits in crops. The seven selected primers produced 34 detectable amplicons in our DNA samples with the mean of 4.8 loci per primer. The number of amplified loci ranged from 1 to 9, with the approximate size range of 300 to 700bp and all used markers showed 100% polymorphism (Table 2). The executed RAPD markers showed 34 (100%) polymorphic loci in all six genotypes this property of the markers was potentially utilized to characterize different species of the family and genotypes of crops. Similar function of RAPD was reported in other valuable crops [30-31]. Observed polymorphic loci could be used as a tool to evaluate genetic diversity of species on the basis of presence and absence of specific locus [32,33], [18]. The least numbers of bands/loci i.e., one was amplified with primer B-18 (CCACAGCAGT), while the maximum number of loci i.e., mine was amplified with primer C-19 (GTTGCCAGCC). Using polymorphic RAPD and ISSR markers, the variability analysis was recorded among the species of *Medicago* [34,35].

During the present study, three separate PCRs were carried out using one arbitrary primer B-18 in first reaction, three primers A-16, C-08 and C-18 in second and three primers C-19, D-10 and E-12 in the third reaction respectively (Table 3).

A similar approach was used and established proper identification system of genotypes and species of *Medicago* and various important plants in the study [36-38], [35]. The characterization of plant species is the most important objective of this study. Many researchers have reported genotype or variety specific loci generated by DNA markers identify verities of Potato [39], *Rhus* species [40] fig varieties [41], *Jatropha* genotypes [42], tea genotypes [43] and *Zingiber officinales* varieties [36].

3.3 Cluster Analysis

The UPGMA method classified six *Medicago* genotypes into different clusters having 61% to 86% homology (Fig. 1). In homology tree, *M. falcata* and *M. lupulina* clustered together having 86% homology while *M. arborea* shared 74% homology with *M. falcata* and *M. lupulina*. *M. polymorpha*, *M. sativa* and TWAL showed different branches with sharing 71%, 63%, 61% respectively homology to other three species (Fig. 1).

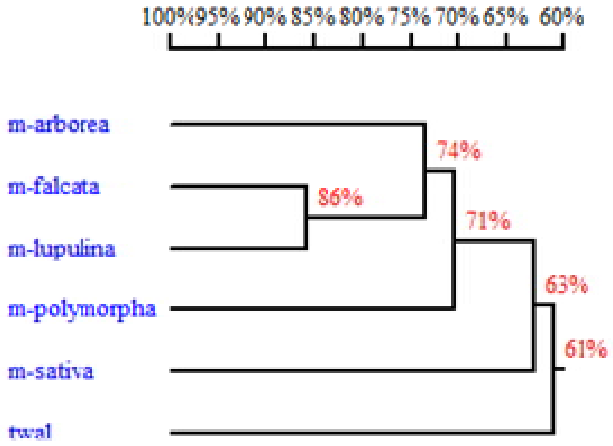

Fig. 1. Homology tree constructed against six accessions of *Medicago* species to determine genetic diversity. To draw a tree, to draw dendogram based on UPGMA, the DNAMAN 5.2.2.0 software was used. In the tree, TWAL*, M. sativa*, *M. polmorphia* and *M. arborea* showed separate branching while *M. falcata* and *M. lupulina* clustered together

Table 1. Bivariate (1-0) data matrix of 6 *Meicago* species using RAPD Primers A-16, B-18, C-08, C-18, C-19, D-10 and E-12

Alleles	TWAL	*M. arborea*	*M. falcate*	*M. sativa*	*M. lupulina*	*M. polymorpha*
1	0	1	0	0	0	0
2	0	0	0	1	0	1
3	0	0	0	1	0	0
4	0	0	0	0	1	0
5	0	0	0	0	0	1
6	0	0	0	1	0	1
7	1	1	0	0	0	0
8	0	0	0	1	0	0
9	0	0	0	0	0	0
10	0	0	1	1	0	1
11	0	1	0	0	0	0
12	1	0	0	0	0	0
13	0	0	1	0	0	0
14	1	1	1	1	1	0
15	1	0	0	1	0	0
16	0	1	0	0	0	0
17	1	0	0	0	0	0
18	1	0	0	0	0	0
19	0	0	0	1	0	0
20	1	1	0	1	0	1
21	1	0	0	0	0	0
22	0	0	0	1	0	0

Table 2. Details of decamer primers used in the present study

Primer name	Sequence (5'-3')	TB	MB	PB	PP%	Range of bands
A-16	AGCCAGCGAA	4	0	4	100%	400-500
B-18	CCACAGCAGT	1	0	1	100%	700
C-08	TGGACCGGTG	7	0	7	100%	350-700
C-18	TGAGTGGGTG	5	0	5	100%	350-700
C-19	GTTGCCAGCC	9	0	9	100%	300-550
D-10	GGTCTACACC	6	0	6	100%	300-600
E-12	TTATCGCCCC	2	0	2	100%	500-600
Total Bands		34	0	34		

Abbreviations: T.B = Total Bands, M.B = Monomorphic Bands, P.B = Polymorphic Bands, PP = Percentage of polymorphism

Table 3. Details of specific loci against different species of *Medicago* generated by selected RAPD primers

Primer	Characteristic band (bp)	Species					
		Arborea	Falcata	Lupulina	Polymorpha	Sativa	TWAL
A16	400	1	0	1	1	0	0
	485	0	0	1	0	0	0
B18	550	0	1	0	0	1	1
C-08	300	0	0	0	0	0	1
	400	1	0	0	0	0	0
	500	0	0	0	0	1	0
	700	0	0	0	1	0	0
C-18	300	0	0	0	0	0	1
	600	1	0	0	0	0	0
	700	0	1	0	0	0	0
Total	11						

Using PCR based DNA markers, genetic diversity was investigated among the species or varieties of alfalfa and different valuable crops [34,35,44,45]. The genetic affinities were investigated based on data generated by RAPD marker and this approach was widely used during previous research [46]. This study could be useful to evaluate taxonomic positions of members of *Medicago* and it is providing a baseline for future to develop different molecular techniques to characterize different species from Pakistan.

4. CONCLUSION

In the present, the polymorphic properties of RAPD marker were utilized and 11 species specific loci were identified. To evaluate genetic diversity of *Medicago* species, cluster analysis was performed and 3 clusters were identified and genetic divergence ranging 61% to 86% among six species was recorded. Here, our study is concluding that *M. falcata* and *M. lupulina*. *M. polymorpha*, *M. sativa* and TWAL had a unique pattern of clustering. This study could be able to provide information based on DNA fingerprints.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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