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Phylogenetic Exploration of *Acacia nilotica*: A Commonly used Medicinal Plant in Himachal Pradesh

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ABSTRACT: *Acacia nilotica* belongs to family, Fabaceae. This family is known to contain highest number of species. The genetic diversity and relationship of six samples of *Acacia nilotica* collected from different geographical regions of Himachal Pradesh, was studied. The regions selected for the analysis were: Solan, Sirmour, Mandi, Bilaspur, Una and Hamirpur. Using RAPD analysis, a total of 12 arbitrary primers were screened while 7 primers showed amplifications. Out of total 68 bands, total 7 monomorphic and 61 polymorphic bands were scored. The percentage of polymorphism shown by the primers was 89%. The genetic diversity was estimated by constructing dendrogram using UPGMA method. The six samples of *Acacia nilotica* were divided into two major clusters. The genotypes of Mandi district showed 44% similarity with the genotypes of Bilaspur district. The genetic similarity between genotypes of Sirmour, Mandi, Bilaspur, Una was 30.2%. The present study revealed that the variation exists among the different accessions of *A. nilotica*, which can be further utilized for the production of quality herbal formulations.

Key words: *Acacia nilotica*, RAPD, genetic variation, polymorphism.

INTRODUCTION

Himachal Pradesh is a state of mountains, expands in the area of 350 to 7,000 m above mean sea level. It is located in the North-West of India. The total geographical area of the state is 55,673 sq km (Chowdhery, 1999). Topographically, the state has been divided into three parts: Shivaliks: also called as outer Himalaya. The regions come under this zone are: Kangra, Una, Hamirpur, Bilaspur, Una, Mandi, Sirmour and Solan districts. The altitude of shivaliks hills ranges from 350-1500 meters above sea level. Mid hills: also called as inner Himalayas. The regions included in these zones are: Churag (Chamba district), Karsog (Mandi district) and Renuka (Sirmour district). The altitude of mid hills ranges from 1500-4500 meters above sea level. Alpine zone: also called as greater Himalayas. The areas come under this zone are, Lahaul-Spiti, Kinnaur, Kullu districts and Pangi tehsil of Chamba district. The altitude of the greater Himalayas starts from 4500 meters above sea level. In a survey of WHO (World Health Organization) it was reported that about 70% of the world's population depend on plant material for their primary health care. About 35,000 to 70,000 plant species have been used for medicinal purposes. Approximately 80% of the population of the developing countries is dependent on the herbal constituents for their primary medical care system (Kim, 2005). Actually the medicinal property is due to the different active principles or constituents present in medicinal plants like alkaloids, flavonoids, phenols, essential oils, steroids and tannins (Anees, 2010). *Acacia* sp. has been reported to contain metabolites *e.g* amines, cyanogenic, fatty acid, alkaloids, seed oils, glycosides, flavonoids, gum, terpenes, tannins and condensed tannins (Seigler, 2003).

From ancient times, *Acacia* sp. have been a good source of tannin, gum, fodder and fuel, therefore these species are well established as an important economic plant. *A. nilotica* is widely used as the source of timber (for making furniture and tools) and firewood species (Gupta, 1970). *A. nilotica*is a multipurpose herbal plant with essential properties that has been widely used for curing of several ailments (Singh *et al*., 2009).

The Indian Himalayan Region (IHR) is known as mega hotspot of biological diversity (Myers, 2000). It comprises about 18% of India and is more than 2,800 km long while 300 km wide. Genetic diversity is the degree of variation in genes among the individuals of a variety, or population of a species (Brown, 1983). It has been studied that genetic variability results from the genetic differences between individuals of a species and may be manifest in variation in DNA sequence in biochemical characteristics. Information about the genetic diversity of species is crucial for sustainable use and efficient conservation efforts of plant genetic resources (PGR) (Spooner *et al*., 2005).

The molecular markers involving techniques provide fast results detailing genetic diversity in species (Souza *et al*., 2008). The genetic diversity of a plant species is an essential element in establishing the conservation strategies (Kim *et al*., 2005).

MATERIAL AND METHODS

A. Plant material

A total of six accessions of *A. nilotica* were collected from six districts of Himachal Pradesh, India. Fresh and young leaf samples were collected and stored in ziplock bags with silica gel and transported back to the laboratory for DNA extraction.

Genomic DNA extraction. CTAB DNA extraction was done by the method of Doyle and Doyle, 1990 with some modifications. 1 gram fresh leaves of selected plant were weighed and crushed into 1000 µl of pre-heated (at 65°C) CTAB DNA extraction buffer (100 mMTrisHCl, 4 M NaCl, 20 mM EDTA, 3% CTAB, 6% PVP and 0.2% β-mercaptoethanol) in an autoclaved 2 ml eppendorf tubes. The samples were given gentle inversions for about 15 minutes. The eppendrof tubes were incubated in a warm water bath (at 65°C) for 30 minutes and regular inversions made after 5-10 minutes. Now, the homogenates were removed from the water bath and kept at room temperature and treated with Tris-Phenol: Chloroform: Isoamyl alcohol (25:24:1, v/v) was added in equal volume to the homogenates. After centrifugation the aqueous phase was carefully pipette out into a sterile new eppendorf tube. The aqueous phase, so obtained was retreated with chloroform: iso-amyl alcohol (24:1) firstly by incubating them for 45 min with regular swirling and then centrifuged at 10,000 rpm (7500 g) at 4°C. Absolute ethanol was added into the eppendorfs for the DNA precipitation, kept at -20°C for overnight. Next day samples were centrifuged at 10,000 rpm at 4°C. DNA was washed twice with 70 % ethanol, re-suspended in TE buffer and stored at −20°C for further use.

Purification of DNA. Purification of the DNA was done by treating it with RNase to remove RNA. 2 µl of RNase was added to nucleic acid extraction and incubated at 37ºC for an hour.

The purity of DNA was determined as = O.D at 260 nm/ O.D at 280 nm (Sambrook *et al*., 1989).

The following formula was used for making dilution:

(STOCK) N1V1 = N2V2 (WORKING)

RAPD primers screening. Screening of the primers is an important step in RAPD-PCR analysis. The decamer oligonucleotides primers were used in this study Primers that gave clear and polymorphic profiles (Table 1) were chosen for further study (D'amato and Corach, 1997).

RAPD PCR amplification. DNA amplification of the six samples of *A. nilotica* was performed in an Automatic Thermal Cycler using RAPD markers. The RAPD reactions were performed with some modifications.

Table 1: Code, sequence and nucleotide length of the primers used in RAPD analysis.

S. No.	Primer code	Primer sequence (5' to 3')	Nucleotide length
1.	OPN 09	TGCCGGCTTG	10-mers
2.	OPB 20	GGACCCTTAC	10-mers
3.	OPN 16	AAGCGACCTG	10-mers
4.	OPA-Y17	GACGTGGTGA	10-mers
5.	OPA-Y15	AGTCGCCCTT	10-mers
6.	OPA-Y05	GGCTGCGACA	10-mers
7.	OPA-Y11	AGACGATGGG	10-mers
8.	OPA-N12	CACAGACACC	10-mers
9.	OPA-Y04	GGCTGCAATG	10-mers
10.	OPA-M05	GGGAACGTGT	10-mers
11.	OPA-01	CAGGCCCTTC	10-mers
12.	OPA-02	TGCCGAGCTG	10-mers

Table 2: Temperature profile for RAPD PCR.

S. No.	Steps	Temperature	Time	No. of Cycles
1.	Initial step	94 ºC	4 min	1
2.	Denaturation	94 ºC	1 min	45
3.	Annealing	37 ºC	1 min	
4.	Elongation	72 ºC	2 min	
5.	Final elongation	72 ºC	10 min	

The PCR was carried out using 25 µl reaction volume (for 1 PCR tube). The master mixture was prepared using 10X PCR buffer, 100 mMdNTP mix, 3U/µl Taq DNA pol, 10 pmoles/µl of primers and 17.50 µl of Mili-Q water per sample. First added reaction mixture to the each PCR tubes which were kept on ice, then genomic DNA was added to it. After preparation of master mixture, PCR tubes were placed in thermal cycler and programed for cycling parameters (Table 2).

Gel electrophoresis. Agrose gel electrophoresis (BIO-RAD horizontal electrophoresis unit) was used for the separation of DNA fragments. All DNA extracts were electrophoresed in 1.2 % agarose gel in TAE buffer. For the genomic DNA, the electrophoretic unit was run for 1 hour at 75 volts while for the visualization of RAPD-PCR bands, it was run for 1 to 2 hours at 65 volts.

RAPD data analysis and scoring. All the RAPD assays were repeated thrice and only the reproducible bands were scored as present (1), absent (0) for each primer genotype combination. The scoring of bands was done manually. The data was feed in the form of binary data matrix. The Pearson's coefficient of similarity was obtained using UPGMA software (unweighted pair group method based on arithmetic mean) to determine the similarity between the different species.

RESULTS

Six individual populations of *Acacia nilotica* were collected from six different areas. In the investigation 20 random decamer oligonucleotide primers from OPA series (operan technology inc., USA) were screened, of these 20 primers, only 7 primers showed reproducible and scorable Bands (Table 3). As shown in Table 4, different fragments, numbers and length of DNA amplification bands were produced from the primers. The total number of amplified fragments with seven primers were 68 and size of amplified fragments ranged from 100 to 1500 bp (Table 4). The study includes the analysis of *A. nilotica* from different geographical locations. The genetic variation among the samples was determined using the RAPD technique. The genetic diversity of some plants were found positively associated with geographical regions. Degree of similarity between the plant samples was determined by using the dendrogram. Then the individuals with genetic similarity were grouped under similar genotypes with close relationships. The genotypes of Mandi district showed 44% similarity with the genotypes of Bilaspur district. The genetic similarity between genotypes of Sirmour, Mandi, Bilaspur, Una was 30.2%.

Table 3: Primers sequences, number of bands, amplified polymorphic fragments and polymorphism by 7 arbitrary primers.

Primers	Sequence (5'to3')	Total no. of bands	Monomorphic bands	Polymorphic bands	% age of polymerphism
OPN-09	TGCCGGCTTG	5	1	4	80%
OPB-20	GGACCCTTAC	11	0	11	100%
OPA-Y15	AGTCGCCCTT	12	1	11	91%
OPN-16	AAGCGACCTG	13	0	13	100%
OPA-Y11	AGACGATGGG	6	2	4	66%
OPA-Y04	GGCTGCAATG	16	0	16	100%
OPA-02	TGCCGAGCTG	5	3	2	40%
Total		68	7	61	89%

Table 4: Size range of the amplified DNA products obtained from six samples of *A. nilotica* using seven decamer RAPD primers.

S. No.	RAPD Primers	Size of band amplified (bp)
1.	OPN-09	300-950
2.	OPB-20	300-980
3.	OPA-Y15	310-1000
4.	OPN-16	100-1500
5.	OPA-Y11	520-1000
6.	OPA-Y04	100-1000
7.	OPA-02	310-1300

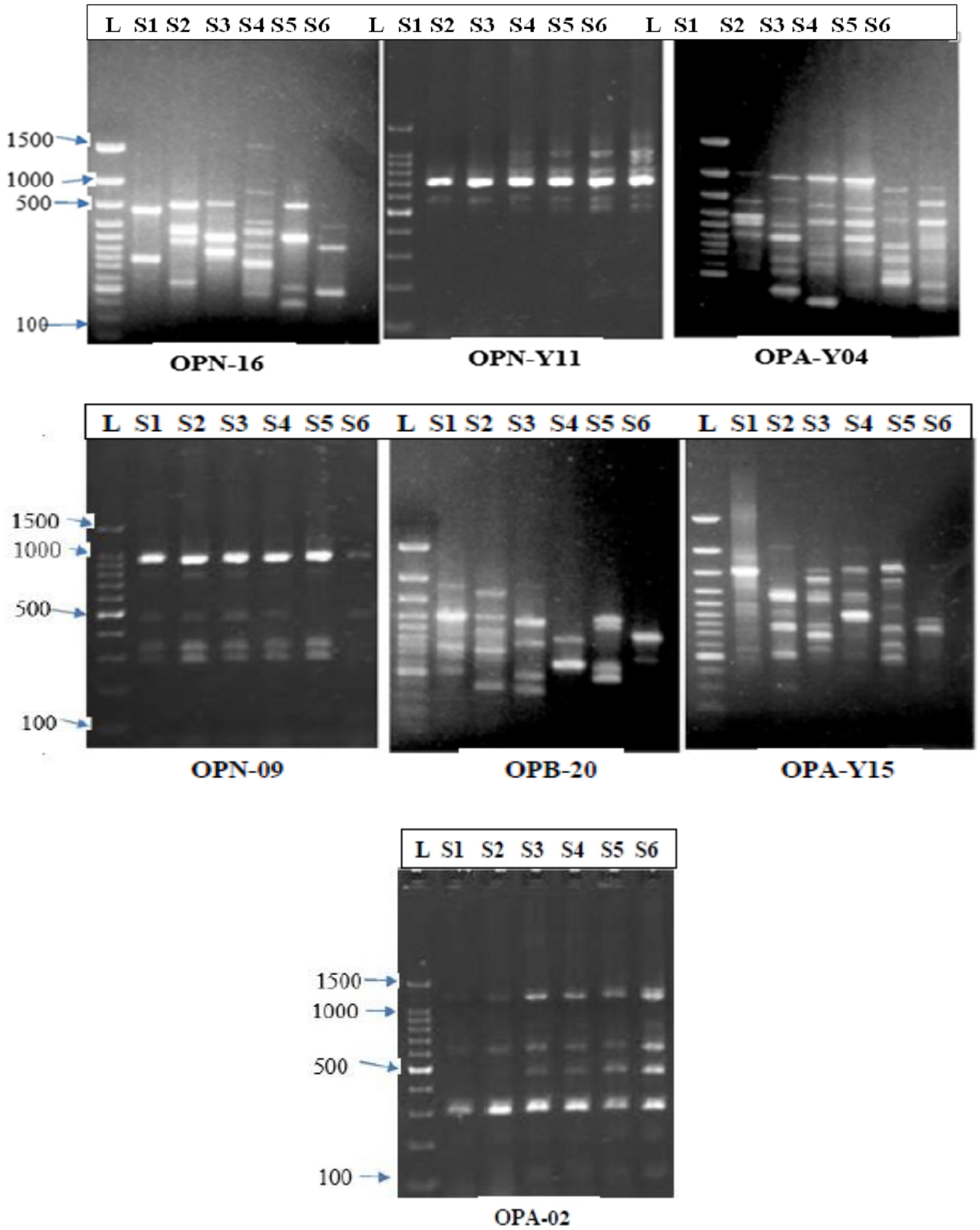

Fig. 1. Banding patterns of RAPD fragments of *A. nilotica* individuals collected from different regions of Himachal Pradesh. L- Ladder, S1-Solan, S2- Sirmour, S3-Mandi, S4-Bilaspur, S5-Una, S6-Hamirpur.

DISCUSSION

The present study is primarily focused on genetic diversity of *A. nilotica* with respect to different geographical locations. The dendrogram obtained through cluster analysis revealed two major clusters (Fig. 2). The first major cluster represented by two districts (Solan and Sirmour). The second major cluster was represented by four districts (Una, Hamirpur, Mandi and Bilaspur). The similarity matrix obtained after multivariant analysis using Pearson's coefficient (Table 5). The results of similarity matrix revealed that the samples from Una district had 49.2%, similarity to the samples from Hamirpur district. The similarity between samples from Una district Mandi district had 48.2% similarity. The samples from Solan and Hamirpur districts were least similar, i.e. 13.8% of each other.

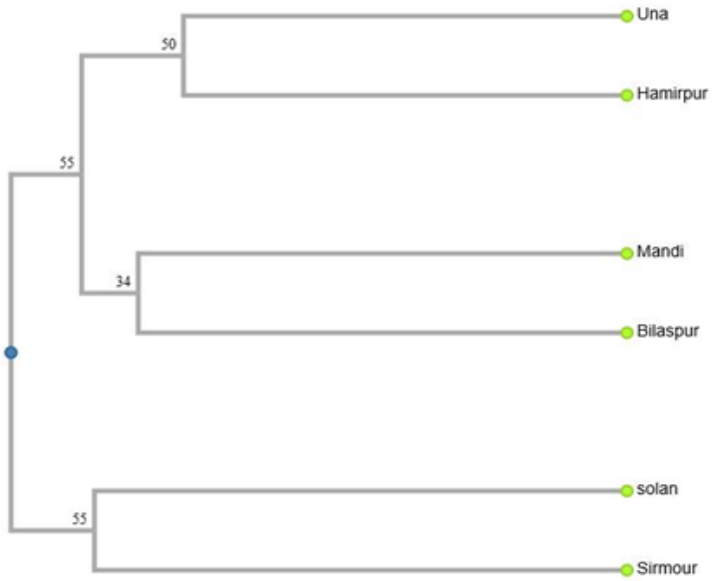

Fig. 2. The phylogenetic tree showing relatedness within species of *A. nilotica* collected from six districts of Himachal Pradesh.

Table 5: Similarity matrix for Pearson's coefficient of six geographical different samples of *A. nilotica.*

	Solan	**Sirmour**	**Mandi**	**Bilaspur**	**Una**	**Hamirpur**
Solan	1	0.390	0.365	0.309	0.309	0.138
Sirmour		1	0.3	0.302	0.302	0.334
Mandi			1	0.44	0.482	0.31
Bilaspur				1	0.316	0.395
Una					1	0.492
Hamirpur						1

Sasikala *et al*. (2015) reported genetic variability in *Terminalia pallida*, collected from six different geographical regions using twenty OPA primers by RAPD analysis. Out of twenty, seven primers showed 33 reproducible and scorable bands. The plant species showed 69.5% polymorphism. Khadidiatou *et al*. (2008) studied the genetic variation in few subspecies of *A. nilotica* of different origins using the RAPD technique. Out of 36 RAPD primers used, only 16 primers produced polymorphism and generated 166 polymorphic bands.

The present study revealed that the variation exists among the different accessions of *A. nilotica*, which can be further utilized for the production of the quality herbal formulations. Based on RAPD analysis, the populations were classified into two clusters. Both the clusters showed the genetic relatedness according to the place of their origin. These observations may be beneficial for quality genetic resources of *A. nilotica* and their sustainable use in medicinal or pharmaceutical industry.

CONCLUSION

A. nilotica have certain important secondary metabolites that confer different pharmacological activities, which can be useful for mankind. This tree is perennial in nature and that may be the reason for this type of variations. For the conservation of a particular plant species the accurate assessment of genetic diversity is an important objective. The present study might be helpful in the field of genetic variability in *A. nilotica*. As results of the present study concluded that *A. nilotica* collected from different geographical areas of Himachal Pradesh possess a certain level of genetic diversity. This study indicated that RAPD is sufficiently informative and powerful technique to detect genetic variability in natural populations of *A. nilotica.* From this study it was concluded that geographical diversity is important and associated with the sole factor of determining the genetic diversity in *A. nilotica*.

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