Population analysis of *Melia azedarach* L. by RAPD markers in the region of Rajasthan.

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BY

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CERTIFICATE

I declare that the dissertation entitled “POPULATION ANALYSIS OF *Melia azedarach* L. BY RAPD MARKERS IN THE REGION OF RAJASTHAN” has been prepared by me under the guidance of Dr. Pankaj Bhardwaj, Assistant Professor, Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

Population Analysis of *Melia azedarach* L. By RAPD markers in the region of Rajasthan.

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Melia azedarach L is ecologically imperative species growing in Thar Desert. Biological effects such as antiviral, anthelminthic, antibacterial, etc. makes it important, yet a little is known about its genetic diversity and structure. In this study, we employed 30 RAPD primers for DNA profiling of 91 individuals representing 8 geographically isolated populations. A total of 98 bands were scored with an average of 3.2 bands per primer. The PIC ranged from 0.0681 to 0.5351 with an average of 0.4103. Nei's genetic diversity (h) and Shannon's information index (I) ranged from 0.1237 to 0.2375 and 0.1845 to 0.3500 respectively with average Nei'sgenetic diversity of 0.2570. The gene flow (Nm) 0.9295 and the genetic diversity of 0.3498 at species level demonstrated overall high level of genetic diversity. Cladistics analysis using DARwin and Bayesian cluster analysis using STRUCTURE placed 44 individuals into two main clusters or original genetic stocks (K = 2) which show little or no association with the geographic origin. The lack of clear assignment of individuals to geographical regions of sampling and consideration of different populations in the same genetic cluster suggests a recent common evolutionary history. *M. azedarach* is a good reforestation tree because of its fast growth and drought hardy nature. This study adds a foundation for more precise inference about the biogeography and management in the reforestation projects in the Thar Desert.

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ABBREVIATIONS

Full form	Abbreviation
Deoxyribonucleic acid	DNA
Cetyltrimethyl ammonium bromide	CTAB
Molar	M
Nanogram	ng
Micro liter	µl
Degree Celsius	^{0}C
Milliliter	ml
Polymerase Chain Reaction	PCR
Deoxynucleoside triphosphates	dNTP
Amplified fragment length polymorphism	AFLP
Random amplified polymorphic DNA	RAPD
Restriction fragment length polymorphism	RFLP
Simple sequence repeats	SSRs
Population geneticanalysis ver. 1.32	POPGENE
Unweighted pair group method with arithmetic averages	UPGMA

Chapter I

Introduction

Melia azedarach L. plant belong to the family Meliaceaee. It is a deciduous tree and commonly known as, Bakain, Dek and Dharek. Branches of Bakain are fleshy, with purplish bark and dotted with buff-colored lenticels. Leaves are compounds, alternate, and without hair. Leaflets are 2-8 cm long, notch, dark green above, often with thin hairs along the veins and light green and generally smooth below. The inflorescence is a panicle from leaf axils and from leafless nodes on the lower part of the new growth. Flowers are fragrant and fruit of the tree is small, yellow drupe, nearly round, smooth and slightly fleshy in nature (Burks, 1997). This species has been widely cultivated as an ornamental and shade tree, as it is well adapted to warm climates, poor soils, and seasonally dry conditions (Harrison *et al.*, 2003). *M. azedarach* plant has been proven to possess several biological effects including antihelminthic, antiviral, antibacterial, antifungal, antiprotozoal, phototoxic, cytotoxic, antiulcer, and immunomodulatory activities (Andrei *et al.*, 1985; Benencia *et al.*, 1994; Khan *et al.*, 2001; Moursi and Al-Khatib, 1984; Palacios *et al.*, 2009).

1.1. Distribution of *M. azedarach*

M. azedarach is found in tropical to sub- tropical temperate climate with a temperature range of -5 to 40^0C within an elevation range of 700 to 1400 m (Yulianti *et al.*, 2011). In Asia this tree typically grows in tropical and subtropical regions (Pakistan, India, and southern Asia), but nowadays it is also cultivated in other warm regions of the world because of its considerable climatic tolerance (Benencia *et al.*, 1994). *M.azedarach* is of very high economic value and is found over a very wide range of habitats in India, including semiarid areas (Juhany, 2011), because soil of semiarid region is rich in organic carbon and macro nutrients like potassium (K), nitrogen (N), phosphorus (P) which are best suited for the growth of *M. azedarach.* It is a fast growing tree species, which can grow easily in the stress conditions (Saiki *et al.*, 1988). Growth rates of *M. azedarach* plants are high and yields of 17.5 m^3/ha/yr have been recorded.

1.2. Genomic diversity

Genomic diversity refers to the variation of genes within species. The genetic diversity enables the population to adapt to its environment and to respond to natural selection. The amount of genetic variation is the basis of speciation and plays a role in the maintenance of diversity at species and community levels. Genetic diversity within a species often increases with environmental variability. Genetic diversity is required for maintaining evolutionary potential in a changing environment, resist pests and avoid the negative consequences of inbreeding (Bawa and Dayanandan, 1998; Newton *et al.*, 1993). Characterization of genetic diversity is a prerequisite for exploitation of genetic resources for plant improvement. Morphological characterization is often faced with the problems of low penetrance and heritability. Molecular markers are highly heritable, are available in high numbers, and often exhibit enough polymorphism to discriminate closely related individuals. Knowing the distribution of diversity within and among populations of a species is important for conservation because it provides useful guidelines for the preservation of genetic diversity within the species as a whole. If a large proportion of the diversity resides among populations, then more populations must be conserved than if each population contains much of the species level diversity (Francisco-Ortega *et al.*, 2000) .Random Amplified Polymorphic DNA (RAPD) is the cheapest and efficient molecular marker in which amplification of unknown DNA segments of genome with single, short and random oligonucleotide primers is carried out (Campbell *et al.*, 1999; Harris, 1995; Samantaray and Maiti, 2010; Waugh *et al.*, 1992). Homologous RAPD fragments are generated in case of same primer which is likely for closely related individuals. RAPDs are dominant markers i.e. they show dominant inheritance and occurrence of only two alleles or provide information about whether the amplification product is present or not. RAPD provides huge information about population structure and genetic diversity (Campbell *et al.*, 1999).

1.4. Statement of the problem

Despite the importance of *M. azedarach* little is known about its genetics. Its wide geographical distribution and varied habitats indicate that there is probably a large

amount of genetic diversity. Random Amplified Polymorphic DNA technique is usually used to show the level of DNA variability among species and also among individuals within species which are closely related, as well as being able to detect the presence of variation of nucleotide arrangement within DNA (Hördegen *et al.*, 2003; Nathan *et al.*, 2006). In the current study RAPD will be used to determine the patterns of genetic diversities of *M. azedarach* in region of Rajasthan as a first step towards gaining better knowledge of genetic diversity in *M. azedarach*. Hence the major objectives of the present study are:

I. Collection of *M. azedarach* leaf samples from various locations of Rajasthan.
II. Evaluation of genetic diversity and population structure by RAPD marker.

Chapter II

Review of Literature

2.1. *Melia azedarach* and its importance

Melia azedarach is member of family *Meliaceaee.* Its common names are Bead tree, Persian lilac, Chinaberry, Mindi, Magrosa tree, Bakain, Dek , Dharek etc. (Coria *et al.*, 2008; Orhan *et al.*, 2012). It grows in regions with elevation 700-1400m above sea level, rainfall 600-1200mm and in well drained soil with pH between 5.5 to 6 (Yulianti *et al.*, 2011) . This is an important tree for reforestation projects because of its fast growth under inhospitable environment. It is also used for roadside plantings and in irrigated plantations (Brown *et al.*, 1993).This plant is highly adaptable to different soil and climatic conditions (Scocchi *et al.*, 2004). In different regions of world this plant is having different chemical and biological activities which may be due to genetic variations (Szewczuk *et al.*, 2003).

2.2. Studies conducted on *M.azedarach*.

The Meliaceaee plant family is known to contain a variety of compounds, which show insecticidal, antifeedant, growth regulating and development modifying properties (Ambrosio and Guerriero, 2002; Greger *et al.*, 2001; Nakatani *et al.*, 2004; Nathan *et al.*, 2006; Nugroho *et al.*, 1999). The effects of compounds, products and extracts obtainable from *M. azedarach* on insects have been reported to have insecticidal activity (Nathan *et al.*, 2006; Senthil Nathan and Sehoon, 2006). In India, it has been known for quite long time, that the tree members of family Meliaceae are good source of folk medications. This fact drew the attentions of many scientists around the world to study the potential contribution of those plants to their efforts in finding a suitable, effective and environment friendly products to control pests and diseases. Extracts of fruits, seeds, leaves of *M. azedarach* have shown many characteristics of medicinal and pesticidal activities against several pathogenic and pest organisms respectively. Olmos and Lavia (2002) carried out genetic analysis of micropropagated *M. azedarach* plants. In this case genomic mutations occurring during micropropagation was revealed using RAPD analysis alongwith chromosomal analysis i.e. RAPD was used to analyse the genetic stability of in vitro micropropagated plants. 10 primers used in this case indicated high variability with a mean value of 0.79. Higher level of variation was observed among micropropagated plants than those used as explants

due to chimerism. In a culture, variations do not accumulate with increase in culture time(Olmos *et al.*, 2002). In 2011, Yulianti and Siregar have carried out genetic analysis of *Melia azedarach* plants by RAPD for seed improvement. In this case, several factors affect level of genetic variability such as mutation, genetic drift, migration, mating system, population size, selection etc. Moderate level of genetic diversity was observed for *Melia azedarach* at community forests or farmland of West Java due to small population size. Population of mindi at West Java having highest level of genetic variation can be used as a source for seed improvement(Yulianti *et al.*, 2011). The genetic diversity of Indian populations and provenances have been documented through isozyme and RAPD studies (Pamidimarri *et al.*, 2010; Shashidhara *et al.*, 2003; Suma and Balasundaran, 2003). The diversity was large among provenances probably because of their geographic isolation but it was very low within provenances probably due to inbreeding caused by genetic erosion.Besides these many researchers have been done on *M. azedarach* L.some of which are listed below in table-2.1.

Table 2.1: List of Research work on *M.azederech*

S.no	Topic of the article/journal	Results or finding	References
1	Effect of *Melia azedarach* L. leaf extracts on human complement and polymorphonuclear leukocytes	The extract of *M.azedarach* L. did not affect the phagocytic activity of polymorphonuclear leukocytes, and the respiratory burst of these cells as measured by the nitro blue tetrazolium reduction assay.	Benencia *et al* 1994
2	Assays of cytotoxicity and antiviral activity of crude and semipurified extracts of green leaves of *Melia azedarach* L.	In this study was concluded that an antiviral factor devoid of toxicity exists in *M. azedarach* L extracts, which exhibited a broad spectrum of antiviral activity.	Andrei *et al*., 1985
3	Evaluation of some wood quality measures of eight-year-old Melia azedarach trees.	The area of sapwood in the breast height cross section of *M. azedarach* trees was 84.3 cm^2, compared with 102.3 cm^2 at the base; however, the wood quality of sapwood was greater at breast height level.	Juhany, 2011
4	Effect of *Melia azedarach* fruits on gipsing-restraint stress-induced ulcers in rats.	In this study, antiulcer effect of the lipid components of *M. azedarach* fruits which is mainly due to the phytosterol fraction.	Moursi and Al-Khatib, 1984
5	Phytotoxic effects of *Melia azedarach* L.(Meliaceaee) fruit extract on weeds and	In this study, *A. sativa* and *S. halepense*, was completely inhibited the root and shoot length. *A. sativa* and *S. halepense* are inactive by	Palacios *et al*., 2009

	crops.	phytotoxic compounds,this compounds are present in *M. azedarach* fruits.	
6	Plant recovery of cryopreserved apical meristem-tips of *Melia azedarach* L. using encapsulation/dehydration and assessment of their genetic stability.	In this study, cryopreservation treatment preserved genetic stability, when it was evaluated using the electrophoretic patterns of nine isozyme systems and RAPD profiles.	Scocchi *et al*., 2004
7	Antiparasitic activity of *Melia azedarach* growing in Argentina.	The antiparasitic activity of the drupe extracts of Melia azedarach L. growing in Argentina was tested against a tapeworm and an earthworm, showing to be better against tapeworms than the standard piperazine phosphate.	Szewczuk *et al*., 2003

2.3. Diversity for sustainable development

Forest and forest products are renewable resources and contribute substantially to economic development. They play a major role in enhancing the quality of the environment. Forests preserve the genetic diversity of living resources, which is necessary to sustain and improve agricultural and forestry production; Forest is the raw material for scientific and industrial innovation. Tropical forests are characterized by a great diversity of tree species and this range of variation provides the basis for selection and improvement of forest products (Wadsworth and Zweede, 2006). Genetic diversity is essential for both the long-term stability and short-term productivity of forest ecosystems. The amount of genetic variation within a species and its distribution within and among populations provides clues to the factors that govern the maintenance of variation, inbreeding and gene flow. Genetic diversity is required for maintaining evolutionary potential in a changing environment, resist pests and avoid the negative consequences of inbreeding (Bawa and Dayanandan, 1998; Newton *et al.*, 1993).

2.4. Genetic and population structure by molecular markers

Molecular markers are highly heritable, are available in high numbers, and often exhibit enough polymorphism to discriminate closely related individuals. Knowing the distribution of diversity within and among populations of a species is important for

conservation because it provides useful guidelines for the preservation of genetic diversity within the species as a whole. If a large proportion of the diversity resides among populations, then more populations must be conserved than if each population contains much of the species level diversity (Francisco-Ortega *et al.*, 2000). Population genetics is the quantitative study of the amount and distribution of genetic variation in populations, and the dynamics of the underlying genetic processes. Description of population genetic structure and its dynamics is based on the analysis of allele and genotype frequencies of simple traits whose transmission follow Mendelian rules of inheritance (Frankel, 1984). The estimate of allele frequencies at a locus from knowledge of genotype frequencies is forthright under the assumptions of the Hardy Weinberg principle. The Hardy Weinberg principle provides the foundation for all population genetic investigations. To characterize population genetic structure, the following parameters that describe and quantify the genetic and geographic variation patterns are usually investigated viz. polymorphisms (P) to describe what proportion of gene loci are variable, average number of alleles per locus (A), average heterozygosity (h) to describe what proportion of all gene loci are heterozygous. Genetic diversity can be studied with a number of molecular markers, but RAPD is preferred because it is relatively easy and cheap method for detecting polymorphism within short duration and requires limited amount of DNA (Brown *et al.*, 1993). Forest trees have been exposed to various geographical disturbances and extreme life history characteristics such as long cycle, greater opportunity for accumulation of mutations and exposure to stresses. Thus, to develop and implement effective genetic improvement and conservation strategies in forest trees, it is necessary to integrate the information drawn from the above mentioned population genetic diversity parameters. The summary of all research work at genomic diversity are listed in table-2.2.

Table 2.2: The summary of research works at genomic diversity.

S.no	Topic of the article/journal	Results or finding	References
1	Liberation: acceptable production of tropical forest timber.	Tropical forests are characterized by a great diversity of tree species and this range of variation provides the basis for selection and improvement of forest products.	Wadsworth and zweede, 2006
2	Global climate change and tropical forest genetic resources.	Genetic diversity is required for maintaining evolutionary potential in a changing environment, resist pests and avoid the negative consequence of inbreeding.	Bawa and Dayanandan, 1998
3	Plant genetic diversity in the Canary Islands: a conservation perspective.	A large proportion of the diversity resides among populations, and then more populations must be conserved than if each population contains much of the species level diversity.	Francisco-ortega *et al*., 2000
4	Genetic perspectives of germplasm conservation.	Population genetic structure and its dynamics are based on the analysis of allele and genotype frequencies of simple traits whose transmission follow Mendelian rules of inheritance. These rules are help in germplasm conservation.	Frankel, 1984
5	Population genetics: Forest conservation genetics.	The hardy Weinberg principle provides the foundation for all population genetic investigations. This principle is help in forest conservation.	Yeh *et al*., 2000
6	Analysis of single protoplasts and regenerated plants by PCR and RAPD technology.	In this study was concluded that both procedures are applicable at all tissue culture stages, from single isolated protoplast to regenerated plants.	Brown *et al*., 1993

2.5. Methods of Genetics diversity study

The use of genetic markers in plant breeding dates back to the beginning of the century when, in peas, Bateson and Punnett (1905) indicated the possibility of genetic linkage, between genes controlling flower petal colour and shape of the pollen grain (Bateson and Punnett, 1905). Prior to 1960's, markers used in plant genetics and breeding were those derived from genes controlling discrete phenotypes of easy visual identification such as dwarfism, chlorophyll deficiencies, flower and seed and their morphology. But these morphological markers are limited in number, expressed only at the whole plant level, greatly influenced by environment and exhibit only a low per cent of polymorphism. This picture began to change in the

1960's with the development of molecular markers based on isozyme polymorphism (Hubby and Lewontin, 1966). which continued to provide simple and inexpensive method of obtaining genetic information in tree species (Grattapaglia and Sederoff, 1994). However, isozymes detect only a fewer number of loci and only a limited subset of isozyme loci can be assayed across the life cycle stages for most tree species. The advent of molecular techniques based on the analysis of DNA polymorphisms radically expanded the frontier area of this study as it mitigated the limitations of morphological and biochemical markers both in terms of numbers available and their genetic properties. With the development and application of molecular markers over the last 20 years, we now know more about the genetic structure of forest tree species and the spatial and temporal dynamics of genetic processes, such as mating and gene flow, than ever before. These molecular markers include Randomly Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSRs), Amplified Fragment Length Polymorphism (AFLP), microsatellites or Simple Sequence Repeats (SSRs), Restriction Fragment Length Polymorphism (RFLP) and Single Nucleotide Polymorphisms (SNPs). Random Amplified Polymorphic DNA technique is usually used to show the level of DNA variability among species and also among individuals within species which are closely related, as well as being able to detect the presence of variation of nucleotide arrangement within DNA (Hördegen *et al.*, 2003; Nathan *et al.*, 2006). RAPD is being successfully used to differentiate species, varieties, cultivars, and clones in many crop plants. RAPD has been successfully applied in differentiating between varieties and clones of *Camellia sinensis* and in evaluating the genetic diversity among elite tea (*Camellia sinensis* var. *sinensis*) accessions (Kaundun *et al.*, 2000). The technique has been used to study genetic diversity in many plant genera such as mahoganies (Chalmers *et al.*, 1997), *Eucalyptus (Keil and Griffin, 1994)*, mango (Schnell *et al.*, 1995), *Populus* spp.(Castiglione *et al.*, 1993) , oil palm (Shah *et al.*, 1994), Norway spruce (Scheepers *et al.*, 1997), *Cacao (Whitkus et al., 1998)*, *Amaranthus (Chan and Sun, 1997)*, cotton (Iqbal *et al.*, 2001) and brassicase (Jain *et al.*, 1994). They have also been used to tag genes of agronomic importance (Hormaza *et al.*, 1994; Michelmore *et al.*, 1991) and to develop genetic maps in

Norway Spruce (Binelli and Bucci, 1994) and *Populus* spp. (Bradshaw Jr *et al.*, 1994). RAPD markers can be effectively used to determine the specificity in plant pathogen interaction and to identify markers linked to a resistant gene of interest within a short time (Kuginuki *et al.*, 1997; Naqvi *et al.*, 1995).

Chapter III

Materials and Methods

3.1. Sample collection and field study

The site for this study is Thar Desert where these plants grow and are adapted to both high and low temperature. Plant samples were collected from Rajasthan region of India. Of the 44 *M. azedarach* samples, 5 were from Jaiselmer, 5 from New pali road, 5 from Gharsana, 4 from Anupgarh, 9 from Raisinghnagar, 4 from Karanpur ,5 from Mahajan and 7 from Lunkaransr. Within each region populations were isolated from each other at a distance of at least 50-100 Km and their size varied from 5-10 plants. Complete information regarding sample collection and location is given in Table 3.1 and Fig 3.1. Fresh leaves of each selected plant were collected and kept inside multiple layers of blotting sheets for transportation to the laboratory.

Table 3.1: *M. azedarach* populations from regions of Rajasthan

Population	Sample Size	Longitude (E)	Latitude (N)
Jaisalmer (Jas)	5	70°21'48.26"E	26°52'23.50"N
New Pali Road (New)	5	72°59'57.12"E	26°13'17.32"N
Gharsana (Ghr)	5	73°04'43.72"E	29°01'18.57"N
Anupgarh (Anp)	4	73°12'34.18"E	29°11'21.07"N
Raisinghnagar (Rai)	9	73°26'56.75"E	29°32'09.01"N
Karanpur (Krn)	4	76°58'14.85"E	26°10'55.44"N
Mahajan (Mah)	5	73°50'12.67"E	28°47'16.13"N
Lunkaransr (Lnk)	7	73°45'18.23"E	28°30'01.75"N
Total	**44**		

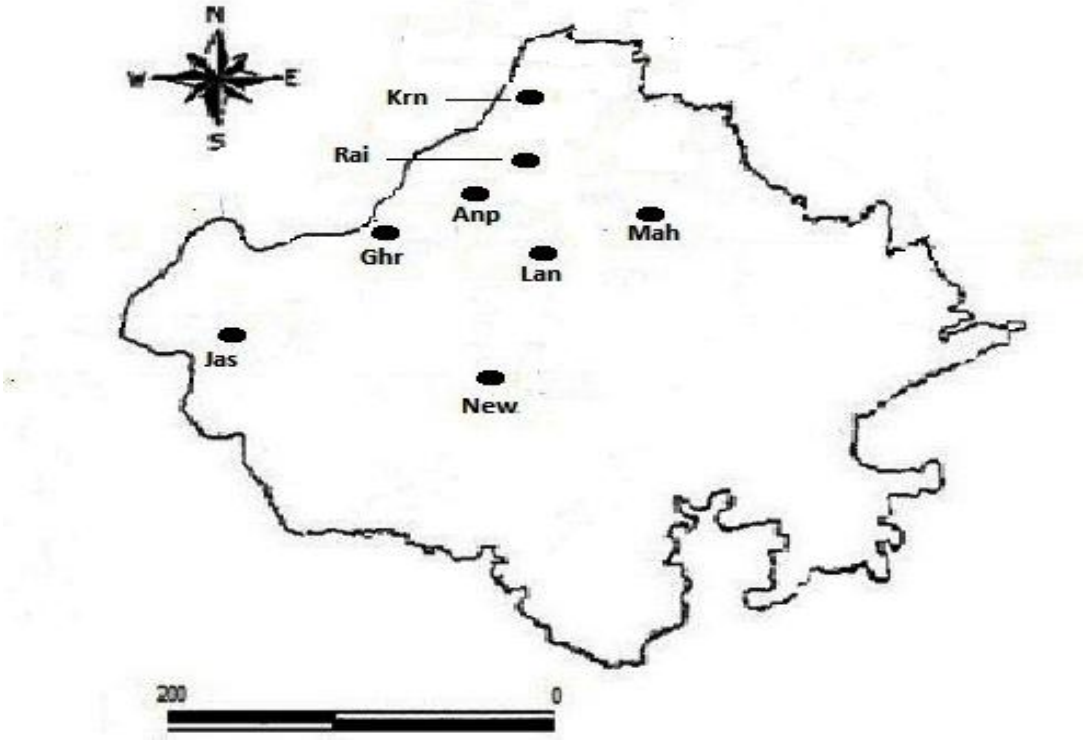

Fig 3.1 Sites of sample collection from Rajasthan region of India

3.2. DNA isolation and purification

Total DNA was extracted from leaf tissue by the CTAB method (Doyle, 1990) with some modifications. The quality and concentration of the extracted DNA were estimated on 0.8% agarose gel and Nano Drop 2000 Spectrophotometer (Appendix A).

3.3. RAPD primers and PCR amplification

Eighty RAPD primers were initially screened for *M.azedarach* DNA amplification and amplification of clear, sharp and repeatable amplicons. Of these, 39 were showing polymorphism with high amplification, 16 primers produced low amplification and 25 primers did not amplify. Out of 39 primers with good amplification 30 were selected.

Genomic DNA was analysed using standard 10 mer oligonucleotide RAPD molecular markers by Williams et al method with some modifications (Williams, 1990). We standardized the DNA amplification conditions by assessing DNA concentration, primer concentration, different bands and concentrations of Taq polymerase as well as temperatures for PCR amplifications. RAPD-PCR amplifications were carried out in a final volume of 20 µl consisting of 25mM $MgCl_2$ solution, 2.5mM dNTPs, 10 picomoles primer, 25ng/ul of genomic DNA, 1.5U of Taq polymerase (Banglore Genei). PCR amplifications were performed in Thermal cycler (Applied Biosystems Veriti 96 well) under the following conditions: Initial denaturation cycle of 2 min at 94°C followed by 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 37°C and extension for 2 min at 72°C with a final extension of 5 min at 72°C. The Amplification products of PCR mixed with 1X loading dye were run on 2% agarose gel prepared in 1X TAE, stained with EtBr at 50V for 90 minutes; then visualized and photographed with the BIORAD Gel Doc X - imager.

3.4. Data analysis

The DNA fragment size was estimated by comparing with DNA size markers (100bp DNA ladder, HiMedia) run on the same gel and DNA fragment size estimated . Amplified loci were scored for presence (1) or absence (0) of bands. Only data from intensely stained, unambiguous, clear bands were included in the analysis. Polymorphism information content (PIC), number of amplified bands, number of polymorphic bands and percentage polymorphism were calculated for each primer which gives estimate about discrimination ability of a marker by considering number of alleles at a locus along with their relative frequencies.

Genetic diversity was estimated using POPGENE software version 1.31 (Yeh *et al.*, 1999) to determine different parameters which include number of polymorphic loci and their percentage, observed number of loci, effective number of alleles($N_e = 1/\sum p_i^2$, where p_i = frequency of the *i*th allele for the studied locus), Nei's genetic diversity ($h= \sum h_k/r$, where h_k = the value of h for the $_k$th locus and r = number of

alleles studied) (Nei, 1973), Shannon's information index and dendrograms.The phylogenetic relationship among populations was generated using software DARwin.

STRUCTURE (version 2.3) (Pritchard *et al.*, 2000) was used to perform a Bayesian analysis to identify hidden population structure by delineating individuals into genetically distinguishable clusters on the basis of their genotypes at multiple loci. The optimal value of K was identified using both the adhoc procedure introduced by Pritchard et al (2000) and method developed by Evanno et al (2005). Population assignment test based on a Bayesian approach was conducted for all the samples using the software STRUCTURE for assigned number of populations of K=1 to 8 and with 3 replicates with a random start for each K value and 1000 runs. The number of distinct population clusters were determined using Δ K method (Evanno *et al.*, 2005).

Chapter IV

Results

4.1. RAPD primers and PCR amplification

Out of the 80 primers that were evaluated in 14 populations, 39 primers were polymorphic and finally 30 primers were chosen on the basis of generation of stable and reproducible bands in all the samples. A total of 98 polymorphic bands were generated with an average of 3.2bands per primer and 0.4103average polymorphism information content (PIC). The highest numbers of polymorphic bands were achieved with primers OPA07, OPB12, OPC15 and OPD02, while the most informative primer was OPC11 with PIC value of 0.5351 (Table 4.1).

Table 4.1: Data of RAPD primers used in the present study and the extent of polymorphism.

S.No.	Primer Name	Primer Sequence(5'-3')	Amplified Bands	Polymorphic Bands	% Polymorphism	PIC
1.	OPA 01	CAGGCCCTTC	1	1	100	0.3752
2.	OPA 02	TGCCGAGCTG	4	4	100	0.4715
3.	OPA 04	AATCGGGCTG	4	4	100	0.4791
4.	OPA 05	AGGGGTCTTG	4	4	100	0.4891
5.	OPA 07	GAAACGGGTG	5	5	100	0.4921
6.	OPA 09	GGGTAACGCC	4	4	100	0.4687
7.	OPA 10	GTGATCGCAG	4	4	100	0.4989
8.	OPA 11	CAATCGCCGT	4	4	100	0.2975
9.	OPA 12	TCGGCGATAG	3	3	100	0.4861
10.	OPA 15	TTCCGAACCC	4	4	100	0.2752
11.	OPA 17	GACCGCTTGT	2	2	100	0.3861
12.	OPA 18	AGGTGACCGT	3	3	100	0.2130
13.	OPB 07	GGTGACGCAG	3	3	100	0.2752
14.	OPB 08	GTCCACACGG	2	2	100	0.4834
15.	OPB 10	CTGCTGGGAC	4	4	100	0.4766
16.	OPB 11	GTAGACCCGT	2	2	100	0.4067
17.	OPB 12	CCTTGACGCA	5	5	100	0.4253
18.	OPB 14	TCCGCTCTGG	4	4	100	0.4529
19.	OPB 15	GGAGGGTGTT	3	3	100	0.4971
20.	OPB 17	AGGGAACGAG	1	1	100	0.0681
21.	OPB 18	CCACAGCAGT	4	4	100	0.4766
22.	OPC 02	GTGAGGCGTC	2	2	100	0.4997
23.	OPC 08	TGGACCGGTG	3	3	100	0.4493
24.	OPC 11	AAAGCTGCGG	4	4	100	0.5351
25.	OPC 12	TGTCATCCCC	4	4	100	0.3831
26.	OPC 15	GACGGATCAG	5	5	100	0.4997
27.	OPC 18	CACACTCCAG	2	2	100	0.1930
28.	OPD 02	GGACCCAACC	5	5	100	0.4976
29.	OPD 03	GTCGCCGTCA	2	2	100	0.3633
30.	OPD 04	TCTGGTGAGG	1	1	100	0.3966

PIC = Polymorphism Information Content

The number of polymorphic loci ranged from 36 (Gharsana and Mahajan) to 70 (New Pali road) within 8 populations with an average of 46.375. The percentages of polymorphic loci ranged from 34.62% (Gharsana and Mahajan) to 67.31% (New Pali Road) with an average of 44.59% (Table 4.2).

Table 4.2: Different populations with Number of polymorphic loci and percentage polymorphic loci

Population	Number of polymorphic loci	percentage of polymorphic loci
Jaisalmer	43	41.35 %
New Pali Road	70	67.31 %
Gharsana	36	34.62 %
Anupgarh	37	35.58 %
Raisinghnagar	66	63.46 %
Karanpur	39	37.50 %
Mahajan	36	34.62 %
Lunkaransar	44	42.31 %

4.2. Genetic variation within populations

Eight parameters were used to assess the genetic variation within populations and they included number of loci or bands, number and percentage of polymorphic loci, actual or observed number of alleles (Na), effective number of alleles (Ne), Nei's genetic diversity (H), Shannon information index (I), expected or genetic diversity within population (intrapopulation diversity for subdivided populations). The highest number of observed alleles (Na =1.6731) were present in New Pali Road population. The highest effective number of alleles (Ne=1.4171), Nei's genetic diversity (H=2.375), Shannon's information index (I=0.3500) was found in Raisinghnagar population (Table 4.3).

Table 4.3: Genetic variability of *M. azedarach* populations detected by RAPD analysis.

Population	Sample Size	Na	Ne	H	I
Jaisalmer (Jas)	5	1.4135	1.3021	0.1680	0.2443
New Pali Road (New)	5	1.6731	1.3609	0.2222	0.3398
Gharsana (Ghr)	5	1.3462	1.2158	0.1237	0.1845
Anupgarh (Anp)	4	1.3558	1.2757	0.1489	0.2148
Raisinghnagar (Rai)	9	1.6346	1.4171	0.2375	0.3500
Karanpur (Krn)	4	1.3750	1.2479	0.1405	0.2079
Mahajan (Mah)	5	1.3462	1.2479	0.1384	0.2019
Lunkaransr (Lnk)	7	1.4231	1.2533	0.1499	0.2247
Total	**44**				

Na = Observed number of alleles, Ne = Effective number of alleles,
H = Nei's gene diversity, I = Shannon's Information index

4.3. Genetic variation among populations

Five parameters were calculated to investigate the genetic variation among populations and these included expected heterozygosity (H_T) or total genetic diversity for all populations, genetic differentiation between subpopulations relative to total genetic diversity ($G_{ST} = 1 - H_S/H_T$ where h_S= population diversity and H_T = total diversity), estimate of gene flow [$Nm = 0.5(1 - G_{ST})/G_{ST}$], Nei's genetic distance and Nei's genetic identity. The average genetic diversity was 0.1661 within population (Hs) and 0.2555 among populations or at species level (H_T). Average estimate of F_{ST} or G_{ST} across all loci were significantly different from zero (average G_{ST} = 0.3498) which indicates limited gene flow and genetic structure in amongst samples (Appendix-B, Table 1). Maximum gene flow was found between population pairs Anupgarh and Raisinghnagar (Nm = 3.0651) followed by New Pali Road and Raisinghnagar (Nm = 2.7694), Gharsana and Raisinghnagar (Nm = 2.7125),

Raisinghnagar and lunkaransar (Nm = 2.7015) and Gharsana and Anupgarh (Nm = 2.5804) (Table 4.4).

Table 4.4: Populations with maximum gene flow.

Population	H_T	H_S	G_{ST}	Nm
Anupgarh & Raisinghnagar	0.2247	0.1932	0.1403	3.0651
New PaliRoad & Raisinghnagar	0.2713	0.2298	0.1529	2.7694
Gharsana & Raisinghnagar	0.2139	0.1806	0.1556	2.7125
Raisinghnagar & lunkaransar	0.2296	0.1937	0.1562	2.7015
Gharsana & Anupgarh	0.1627	0.1363	0.1623	2.5804

According to Nei's unbiased measures of genetic distance (1978) data. the highest genetic distance pairs were between New pali road and Gharsana (0.1938), New pali road and Jaisalmer (0.1664), New pali road and Anupgarh (0.1749) and New pali road and Mahajan (0.1301). Therefore, it indicates New pali road population to be the most differentiated of all the populations. Data of Nei's (1978) Unbiased measures of genetic identity validates the data of genetic distance and the highest genetic identity pairs were between Anupgarh and Gharsana (0.9584), Mahajan and Lunkaransar (0.9465), Anupgarh and Raisinghnagar (0.9437), Gharsana and Raisinghnagar (0.9369) and Mahajan and Karanpur (0.9343). Therefore it indicates that Anupgarh, Gharsana, Mahajan, Raisinghnagar, Karanpur and Lunkaransar are highly similar on genetic level that is these populations are showing strong relationships between them (Appendix – C, Table 1).

4.4. Cladistic analysis

Dendrogram was drawn by Dendrogram based on Neis's genetic distance method (UPGMA modified from Neighbor procedure of PHYLIP version 3.5) to visualize the relationships among 8 populations of *M. azedarach* L. (Fig 4.1). The results represented two clusters, one consisting of the 4 populations (Jaisalmer, Gharsana, Anupgarh and Raisinghnagar) and the other consisting of the other 4 populations. The highest values of Nei's genetic distance are of New Pali road population (0.1938)

and Jaisalmer (0.16664) which is geographically distant from other populations (Appendix-C, Table 1)

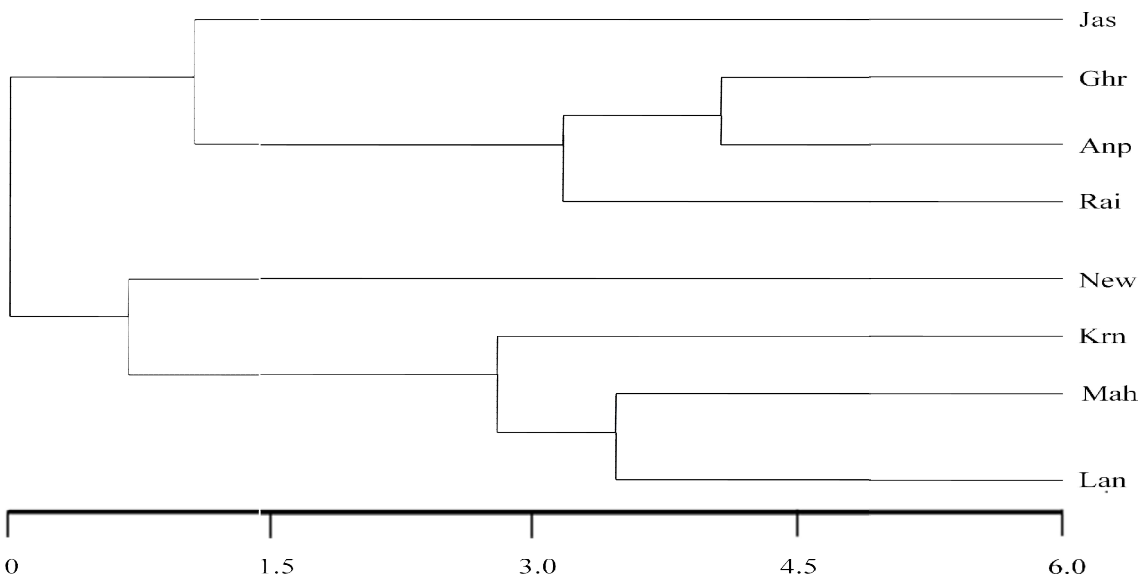

Fig 4.1 Dendrogram based on Nei's (1978) genetic distance

4.5. Ancestry analysis of the planted individuals

STRUCTURE version 2.3 based on Bayesian approach was applied to search for hidden population structure among genotypes of different populations of *M. azedarach.* The relative distinctiveness of sampled populations was determined by assigning individuals on the basis of genetically homogeneous groups rather than on geographical locations of sampling. The highest value of ΔK identified 2 clusters or genetic stocks that were present among 8 sampled populations. This grouping is consistent with UPGMA dendrogram. Graphical method used to detect true number of populations i.e. K indicated mostly the number of genetic stocks at K=2 considering ΔK distribution (Fig 4.2).

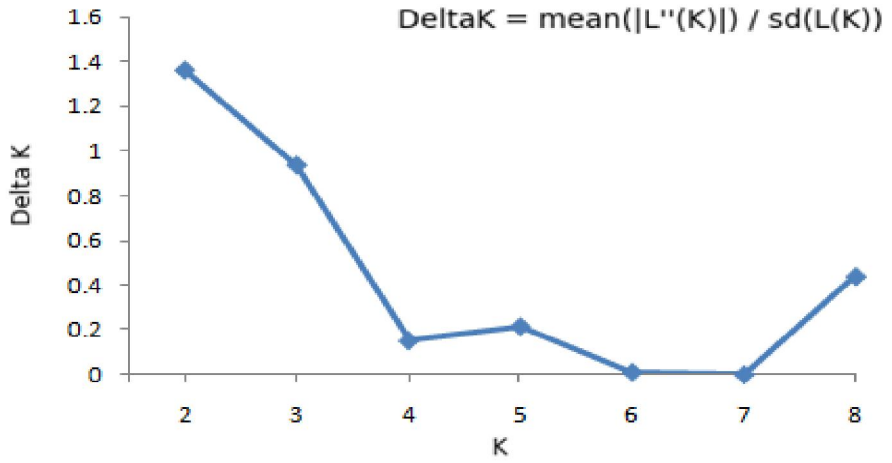

Fig 4.2 Value of K from a range of 1-8 populations using the second order statistics (Δ K) given by Evanno et al. (2005).

Out of 44 individuals, 23 individuals were observed in the admixed stock and 19 and 2 individuals were in the two original genetic stocks (Fig 4.3).

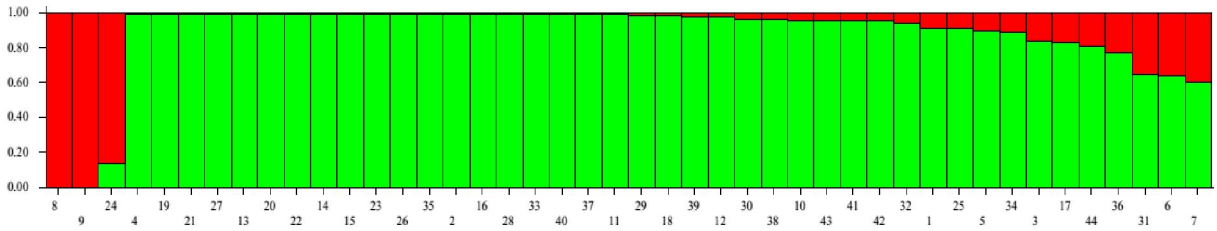

Fig 4.3 Structure plot with membership coefficient of individuals on y-axis. assignment of all 44 individuals into two clusters. Each vertical line represents one individual. Membership coefficient of an individual for a subgroup represents fraction of its genome that has ancestory in subgroup. Two clusters inferred by Structure in combined clustering of all individuals. Each colour represents different genetic stock.

Of the 44 individuals sampled, first smaller genetic cluster occupied 4.55% (2 individuals from 1 populations), second larger genetic cluster occupied 43.18% (19 individuals from 6 populations) and the admixed cluster occupied 52.27% (23 individuals from 8 populations) (Fig 4.4).

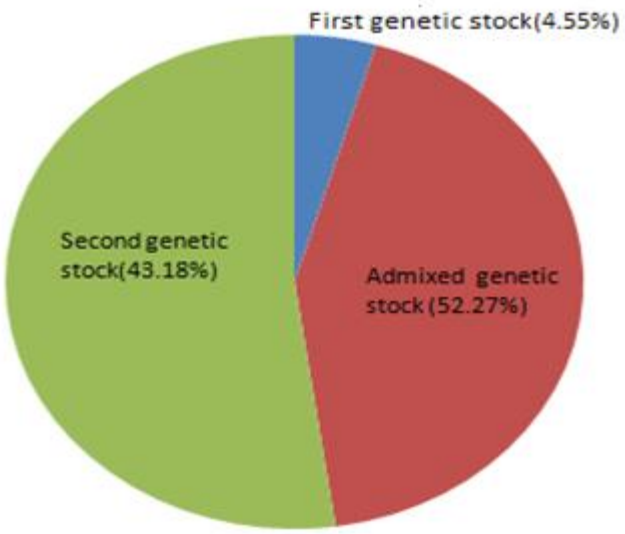

Fig 4.4 Pie chart representing two original and one admixed genetic stock revealed by software STRUCTURE 2.3 version.

DARwin software was employed to construct the unrooted tree of all 8 populations. Each coloured branch represents one individuals collected from corresponding inferred population (Fig 4.5).

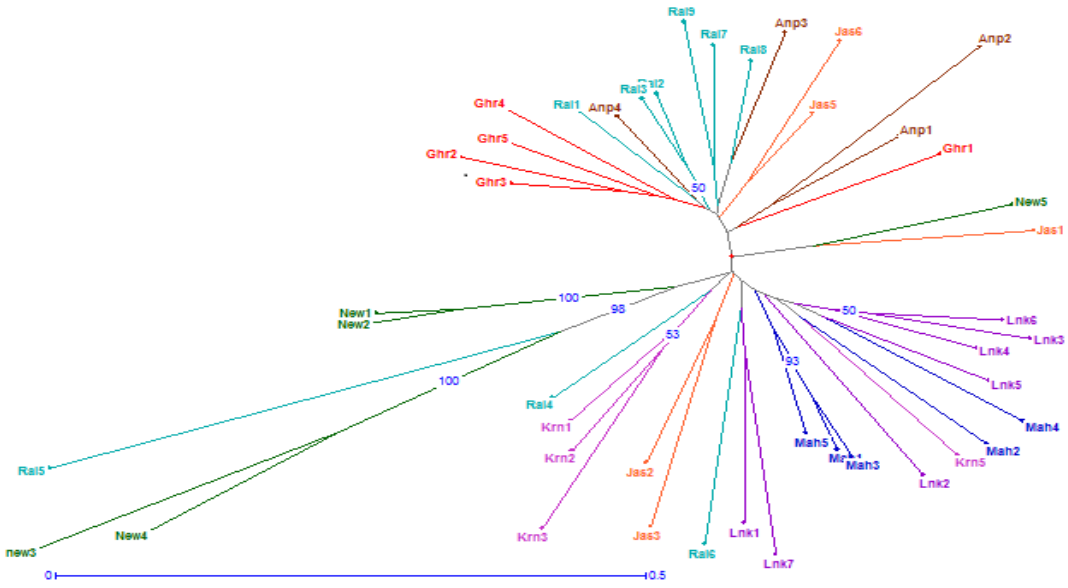

Fig 4.5 Unrooted tree construted by DARwin. Each coloured branch represents one individual collected from corresponding inferred population.

Chapter V
Discussion

Genetic diversity, a basic source of biodiversity is the total number of traits leading to variation within or among populations of a species (Poczai *et al.*, 2012). Maintenance of genetic diversity and population distinctiveness plays an important role in biodiversity and conservation of species as it helps to understand the processes or factors involved in genetic variation in populations and species (Nongrum *et al.*, 2012).

RAPD has been used to detect genetic diversity within and between populations of *Populuseu phratica* (Saito *et al.*, 2002)*, Vitellaria paradoxa* (Fontaine *et al.*, 2004), *Gliricidia sepium* (Dawson *et al.*, 1995), *Mangifera indica* (Díaz-Matallana *et al.*, 2009)and many others. High values of percentage polymorphism for all the primers indicated that the main cause for high genetic diversity may be geographic isolation which regulates the process of genetic diversity and variation. High level of genetic diversity due to geographic isolation in *Anethum graveolens* populations and RAPD was used to reveal its genetic structure (Suresh *et al.*, 2013). The highest value of percentage of polymorphic loci is of New Pali road population (67.31%) which is geographically distant from other populations and dendrogram agrees with this data. This suggested that New Pali road population was the most distant and differentiated population. Present investigation using RAPD marker revealed that in *M. azedarach* highest genetic variation was observed among populations (H_T = 0.2555) as compared to within population (H_S = 0.1661). Till now investigations regarding genetic diversity revealed low levels of genetic *diversity* within populations in case of populations of geographically restricted plant species but high levels of genetic diversity within population was observed in case of out-crossing species (Torres *et al.*, 2003; Wagner *et al.*, 2011).When genetic diversity is classified into within and among population genetic diversities, selfing species exhibit low levels of genetic diversity within population, but a considerable high genetic diversity among populations (He *et al.*, 2007).

Genetic diversity among the populations and geographic range are highly related (Warghat *et al.*, 2012). Several factors like geographical isolation, population fragmentation, breeding system and genetic drifts may be responsible for high

population differentiation (Hogbin and Peakall, 1999; Zong *et al.*, 2008). F_{ST} values ranging from 0 to 0.05 represent little or very little genetic diversity, from 0.05 to 0.15 represent considerable genetic diversity and that >0.25 represent very high genetic diversity. The results of average F_{ST} (0.3498) indicated very high genetic diversity among populations. If populations are small and isolated from one another, the genetic drift could be capable of influencing the genetic structure and increasing differentiation among populations (Ellstrand and Elam, 1993). High genetic diversity indicated limited gene flow among *M. azedarach* populations which agrees with estimate of gene flow (Nm) obtained with POPGENE (version 1.31). Nm represents historical average levels of gene flow. A G_{ST} derived average genetic flow (Average Nm = 0.9295) revealed that gene flow between populations of *M. azedarach* is restricted and it is below the level (Nm>4) required to counteract genetic drift (i.e. Nm>4 according to stepping stone model) (Slatkin, 1993). But in some population pairs Nm value revealed high gene flow.

If Nm>1 (in an infinite island model) or Nm>4 (in a stepping stone model), the gene flow is quite sufficient to create genetic differentiation between populations balanced for migration and genetic drift. But according to infinite island model, if 0.5>Nm>1, genetic differentiation among populations is small but considerable in case of stepping stone model (Díaz-Matallana *et al.*, 2009). In the present study average Nm = 0.9295 which indicates limited gene flow among populations and agrees with the average F_{ST} value (0.3498) because F_{ST} is inversely related to Nm, the effective migration rate. A high F_{ST} indicates that populations are genetically differentiated the result of a low migration of genotypes.

Maximum gene flow was found between population pairs Anupgarh and Raisinghnagar (Nm = 3.0651) followed by New Pali Road and Raisinghnagar (Nm = 2.7694), Gharsana and Raisinghnagar (Nm = 2.7125), Raisinghnagar and lunkaransar (Nm = 2.7015) and Gharsana and Anupgarh (Nm = 2.5804) (Table 4.4). All the other population pairs had Nm values <4 which indicates low gene flow (according to stepping stone model). These values agree with results of Nei's unbiased genetic identity and distance. Some of these populations are

geographically closer and some are quite distant. This indicates that geographically closer populations undergo high gene flow when migrant genes arriving by pollen or seed or human interference become established in new genets (Ayres and Ryan, 1999). Pollen dispersal by wind and insects between populations may be affected by environmental conditions, geographic distances between populations and topographic conditions (Islam *et al.*, 2012). In the present study high gene flow was observed between populations which were geographically distant from each other. This may be due to human interference. Long distance seed dispersal by humans has been observed as a source of gene flow among populations of Italian wild Cherry (*Prunusavium* L.) (De Rogatis *et al.*, 2013).

Clustering of different populations of *M. azedarach* in dendrogram based on Nei's genetic distance was not completely in congruent with their geographic locations and so it indicates that genetic diversity is not corroborating with geographical diversity. The data strongly suggests that overall genetic diversity is high and gene flow is limited but in some population pairs G_{ST} value is very low that is low genetic diversity and high Nm value (Nm>4) that is high gene flow. Overall high genetic diversity may be due to geographic isolation, habitat fragmentation or mating system but high gene flow in some population pairs may be due to human interference, pollen or seed dispersal etc.

Cluster analysis in *M. azedarach* showed that genetic diversity based on morphological traits was not in accordance to geographical regions (Solouki *et al.*, 2008). There was no clear clustering pattern of geographically closer individuals. Same results were obtained in the case of *Anethum graveolens L.* (Suresh *et al.*, 2013). These observations clearly indicated that the association between genetic similarity and geographical distance was less significant. These genetic differences among populations may be due to selection, adaptation, migration, genetic drift and method of pollination. The other factor of importance is also vitally related to the environment and human interference (Solouki *et al.*, 2008). Dendrogram and cluster analysis showed that some landraces with same geographical area were clustered into different groups and no preferred relationship existed between geographical

distribution and the dendrogram. Investigations related to cluster analysis have found clusters including trees from different populations which indicated that plants can be more similar to those from other populations than to those of their own population in case of *Ilex paraguariensis* (Gauer and Cavalli-Molina, 2000).

Both of the original genetic stocks consisted of individuals of populations from different geographical locations i.e. the two genetic clusters identified by STRUCTURE were not according to the geographical locations of sampling. This represented that a high level of gene flow has occurred. Structure infers the highest likelihood of both the individual clusters and the admixture of genotypes using allele frequency and linkage disequilibrium information from dataset directly. The Bayesian structure analysis, dendrogram and unrooted tree (Fig 4.5.) did not group. 8 *M. azedarach* populations may have originated from two ancestral gene pools and the presence of admixed cluster or stock suggested the occurrence of gene flow. None of the clusters determined by STRUCTURE either original or the admixed one consisted of specified full populations which indicated high gene flow.

Gene flow at the species level was limited due to wide geographical distribution or restricted seed and pollen dispersal. Gene flow and life history traits such as population size change, habitat fragmentation, bottlenecks etc. have been considered as the main determinants of population genetic structure. Therefore different populations considered in the same genetic clusters, suggests recent common ancestry to be the important factor rather than other events leading to gene flow.

SUMMARY

M. azedarach L is an ecologically important species for the ecosystem of Thar Desert. It is economically important due to its wood quality and several biological effects, yet a little is known about its genetic diversity and structure. Genetic diversity is a necessary requirement for the improvement, use and conservation of plant genetic resources.

RAPDs markers were used in this study because they allow estimation of genetic diversity among organisms with unknown previous genetic information. In fact, the investigation is the first report on the structure of the genetic diversity of *M. azedarach* L in the region of Rajasthan (India). Genetic polymorphism detected with RAPD reveals one allele per locus which corresponds to the amplification product visualized and polymorphic bands were generated with an average of 3.2 bands per primer. High values of percentage polymorphism for all the primers indicated that the main cause for high genetic diversity may be geographic isolation which regulates the process of genetic diversity and variation. Population wise highest value of percentage of polymorphic loci is of New Pali road population which is geographically distant from other populations and dendrogram agrees with this study. This suggested that New Pali road population was the most distant and differentiated population.

Present investigation using RAPD marker revealed that in *M. azedarach* highest genetic variation was observed among populations as compared to within population. Till now investigations regarding genetic diversity revealed low levels of genetic *diversity* within populations in case of populations of geographically restricted plant species but high levels of genetic diversity within population was observed in case of out-crossing species. This study strongly suggests that overall genetic diversity is high and gene flow is limited but in some population pairs G_{ST} value is very low that is low genetic diversity and high Nm value (Nm>4) that is high gene flow. Overall high genetic diversity may be due to geographic isolation, habitat fragmentation or mating system but high gene flow in some population pairs may be due to human interference, pollen or seed dispersal etc. Moreover, the high genetic

diversity within populations is explained by the breeding system since *M. azedarach* L is an allogamous species.

Gene flow at the species level was limited due to wide geographical distribution or restricted seed and pollen dispersal. Gene flow and life history traits such as population size change, habitat fragmentation, bottlenecks etc. have been considered as the main determinants of population genetic structure. Therefore, it was concluded that the different populations considered in the same genetic clusters, suggests recent common ancestry to be the important factor rather than other events leading to gene flow.

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APPENDIX- A

DNA isolation protocol

DNA was isolated following the protocol of Doyle 1990; as:

- 5g of plant material were homogenized in liquid nitrogen with help of pre-cooled mortar and pestles and mixed with pre-heated DEB at 65^{o}C.
- The powder was transferred to 25 ml polypropylene centrifuge tube containing 10 ml of pre-warmed (65^{o}C) DNA extraction buffer and suspension was incubated for I hour at 65^{o}C and cool it to room temperature.
- The mixture was emulsified with an equal volume of chlorophorm:isoamylalcohol (24:1) for 5 min by gentle inversion.
- Make the final volume in each polypropylene centrifuge tube equal by adding CIA.
- The mixture was centrifuged at 15,000 rpm for 30 min.
- The aqueous phase (supernatant) was transferred to a fresh centrifuge tube with a wide bore pipette and add 2/3 volume of iso-propanol to it by quick and gentle inversion.
- The precipitated DNA was spooled out using disposable pipette tip.
- Washed twice with 70% alcohol at 13,000rpm for 5 min.
- The pellet was dried under vacuum and dissolved in 1ml of $T_{10}E_1$ buffer and incubated at 37ºC.

DNA purification protocol

- Purification of DNA is needed to remove RNA, proteins and polysaccharides, which are considered to be the major contaminants in the DNA precipitates. RNA was removed by RNAse treatments and proteins were removed by phenol-chloroform extraction.
- 10µl RNase A (1µg/1µl) is added to the DNA sample (500µl) and incubated at 37°C for 1 hr.
- After 1 hr equal volume of phenol: chloroform: iso-amyl alcohol (25:24:1) was added and tube were spun at 10,000 rpm for 5 min at the room temperature. The aqueous phase was separated into fresh micro centrifuge tube.
- The upper aqueous phase was collected and extracted with chloroform–isoamyl alcohol for the second time.
- Extracted aqueous phase is further purified by adding equal volume of Chloroform:Isoamyl(24:1) and centrifuged at10,000 rpm for 5 min.
- The above step is repeated again.
- The separated upper aqueous phase was collected after centrifugation and mixed with 1/10th volume of 3 M sodium acetate.

- DNA was precipitated by adding two volumes of absolute alcohol, pelleted by centrifugation at 13,000 rpm for 3 minutes.
- The pellet is then washed with 70% alcohol twice at 10,000 for 3 min. and dried in vacuum and dissolved in $T_{10}E_1$ buffer.

APPENDIX- B

Table 1: Nei's genetic diversity analysis

Locus	Sample Size	H_T	H_S	G_{ST}	Nm
OPA01-1	44	0.4898	0.3503	0.2848	1.2558
OPA01-2	44	0.3427	0.1540	0.5505	0.4083
OPA01-3	44	0.4996	0.2926	0.4143	0.7068
OPA01-4	44	0.4998	0.2748	0.4503	0.6105
OPA02-1	44	0.1490	0.1371	0.0801	5.7392
OPA02-2	44	0.1395	0.1188	0.1484	2.8688
OPA02-3	44	0.4828	0.3314	0.3135	1.0948
OPA02-4	44	0.4643	0.3496	0.2470	1.5241
OPA04-1	44	0.4881	0.3443	0.2945	1.1976
OPA04-2	44	0.4511	0.3944	0.1256	3.4805
OPA04-3	44	0.1992	0.1641	0.1759	2.3427
OPA04-4	44	0.4475	0.2798	0.3747	0.8343
OPA05-1	44	0.4495	0.2888	0.3577	0.8980
OPA05-2	44	0.4847	0.2558	0.4722	0.5589
OPA05-3	44	0.3132	0.1610	0.4859	0.5289
OPA05-4	44	0.0548	0.0436	0.2029	1.9637
OPA07-1	44	0.1286	0.0618	0.5196	0.4623
OPA07-2	44	0.0548	0.0436	0.2029	1.9637
OPA07-3	44	0.2746	0.2466	0.1021	4.3960
OPA07-4	44	0.4968	0.2520	0.4928	0.5146
OPA09-1	44	0.4717	0.2815	0.4031	0.7403
OPA09-2	44	0.0858	0.0727	0.1533	2.7619
OPA09-3	44	0.0260	0.0236	0.0936	4.8412
OPA09-4	44	0.4813	0.3401	0.2933	1.2048
OPA10-1	44	0.2859	0.2226	0.2213	1.7597
OPA10-2	44	0.4453	0.2877	0.3539	0.9130
OPA10-3	44	0.0142	0.0135	0.0504	9.4202
OPA10-4	44	0.0260	0.0236	0.0936	4.8412
OPA11-1	44	0.4989	0.3841	0.2302	1.6718
OPA11-2	44	0.0329	0.0290	0.1192	3.6938
OPA11-3	44	0.2699	0.2145	0.2054	1.9348
OPA11-4	44	0.3503	0.2584	0.2624	1.4055
OPA12-1	44	0.4997	0.1607	0.6784	0.2370
OPA12-2	44	0.1553	0.1430	0.0794	5.7954
OPA12-3	44	0.3347	0.2842	0.1509	2.8128
OPA12-4	44	0.0581	0.0526	0.0943	4.8000
OPA15-1	44	0.1064	0.0873	0.1791	2.2910
OPA15-2	44	0.4473	0.2919	0.3473	0.9395
OPA15-3	44	0.0000	0.0000	0.0000	0.0000
OPA15-4	44	0.0793	0.0673	0.1521	2.7873
OPA17-1	44	0.0291	0.0260	0.1049	4.2678

OPA17-2	44	0.1296	0.1224	0.0553	8.5351
OPA17-3	44	0.1821	0.1528	0.1608	2.6096
OPA17-4	44	0.0826	0.0762	0.0768	6.0129
OPA18-1	44	0.0000	0.0000	0.0000	0.0000
OPA18-2	44	0.3808	0.1614	0.5762	0.3677
OPA18-3	44	0.2595	0.2243	0.1354	3.1938
OPA18-4	44	0.1296	0.0760	0.4137	0.7086
OPB08-1	44	0.4962	0.3163	0.3626	0.8789
OPB08-2	44	0.4754	0.4306	0.0943	4.8029
OPB08-3	44	0.0000	0.0000	0.0000	0.0000
OPB08-4	44	0.0548	0.0436	0.2029	1.9637
OPB11-1	44	0.1711	0.1388	0.1884	2.1540
OPB11-2	44	0.4861	0.2404	0.5055	0.4892
OPB11-3	44	0.4104	0.1901	0.5368	0.4314
OPB11-4	44	0.1533	0.1374	0.1037	4.3215
OPB12-1	44	0.4992	0.2959	0.4072	0.7278
OPB12-2	44	0.4467	0.1998	0.5527	0.4046
OPB12-3	44	0.0184	0.0172	0.0655	7.1319
OPB15-1	44	0.4992	0.3952	0.2084	1.8997
OPB15-2	44	0.1265	0.1101	0.1296	3.3576
OPB15-3	44	0.0260	0.0236	0.0936	4.8412
OPB17-1	44	0.4556	0.2770	0.3920	0.7756
OPB17-2	44	0.4960	0.3781	0.2377	1.6036
OPB17-3	44	0.4447	0.2001	0.5501	0.4089
OPB18-1	44	0.0548	0.0436	0.2029	1.9637
OPB18-2	44	0.0000	0.0000	0.0000	0.0000
OPB18-3	44	0.3508	0.1510	0.5696	0.3778
OPC12-1	44	0.0260	0.0236	0.0936	4.8412
OPC12-2	44	0.4794	0.3441	0.2821	1.2721
OPC12-3	44	0.4594	0.2623	0.4291	0.6652
OPC11-1	44	0.4451	0.2660	0.4025	0.7422
OPC11-2	44	0.0142	0.0135	0.0504	9.4202
OPC11-3	44	0.1866	0.1181	0.3674	0.8608
OPC18-1	44	0.0000	0.0000	0.0000	0.0000
OPC18-2	44	0.1761	0.1174	0.3336	0.9990
OPC18-3	44	0.0799	0.0556	0.3043	1.1429
OPC02-1	44	0.0548	0.0436	0.2029	1.9637
OPC02-2	44	0.2939	0.2599	0.1154	3.8322
OPC02-3	44	0.1756	0.1055	0.3996	0.7514
OPC08-1	44	0.0548	0.0436	0.2029	1.9637
OPC08-2	44	0.0000	0.0000	0.0000	0.0000
OPC08-3	44	0.4231	0.2343	0.4463	0.6203
OPC15-1	44	0.0399	0.0371	0.0696	6.6794
OPC15-2	44	0.4759	0.2570	0.4599	0.5872
OPC15-3	44	0.0544	0.0496	0.0866	5.2708
OPD02-1	44	0.0548	0.0436	0.2029	1.9637
OPD02-2	44	0.2795	0.1724	0.3830	0.8053
OPD02-3	44	0.4807	0.2899	0.3970	0.7596
OPD03-1	44	0.4416	0.2965	0.3286	1.0217
OPD03-2	44	0.0260	0.0236	0.0936	4.8412
OPD03-3	44	0.4996	0.0992	0.8014	0.1239
OPD04-1	44	0.0000	0.0000	0.0000	0.0000
OPD04-2	44	0.4987	0.2677	0.4632	0.5794
OPD04-3	44	0.4297	0.2615	0.3913	0.7777
OPB07-1	44	0.0000	0.0000	0.0000	0.0000

OPB07-2	44	0.2431	0.1789	0.2643	1.3921
OPB07-3	44	0.4346	0.2622	0.3965	0.7609
OPB14-1	44	0.0000	0.0000	0.0000	0.0000
OPB14-2	44	0.1056	0.0618	0.4145	0.7063
OPB14-3	44	0.0000	0.0000	0.0000	0.0000
OPB10-1	44	0.4418	0.3294	0.2546	1.4641
OPB10-2	44	0.4916	0.3265	0.3359	0.9887
OPB10-3	44	0.2688	0.2230	0.1702	2.4371
Mean	**44**	**0.2555**	**0.1661**	**0.3498**	**0.9295**
St. Dev		**0.0372**	**0.0151**		

APPENDIX- C

Table 1: Nei's unbiased measures of genetic identity and genetic distance (1978).Nei's genetic identity (above diagonal) and genetic distance (below diagonal). (POPGENE version 1.31).

Pop ID	Jas	New	Ghr	Anp	Rai	Krn	Mah	Lnk
Jas	****	0.8467	0.8763	0.9061	0.9143	0.8903	0.8981	0.8939
New	0.1664	****	0.8238	0.8395	0.9152	0.9092	0.8780	0.8903
Ghr	0.1321	0.1938	****	0.9584	0.9369	0.8773	0.8561	0.8604
Anp	0.0986	0.1749	0.0424	****	0.9437	0.8697	0.8832	0.8711
Rai	0.0896	0.0886	0.0652	0.0579	****	0.9205	0.9164	0.9272
Krn	0.1162	0.0952	0.1309	0.1396	0.0828	****	0.9343	0.9305
Mah	0.1074	0.1301	0.1554	0.1242	0.0874	0.0680	****	0.9465
Lnk	0.1121	0.1162	0.1504	0.1380	0.0756	0.0721	0.0550	****

Trees - Structure and Function

Genetic diversity and population structure of Melia azedarach L. growing in Indian Thar Desert.

--Manuscript Draft--

Manuscript Number:	
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Abstract:	Melia azedarach L is ecologically imperative species growing in Thar Desert. Biological effects such as antiviral, anthelminthic, antibacterial, etc. makes it important, yet a little is known about its genetic diversity and structure. In this study, we employed 30 RAPD primers for DNA profiling of 91 individuals representing 14 geographically isolated populations. A total of 104 bands were scored with an average of 3.5 bands per primer. The PIC ranged from 0.1898 to 0.5000 with an average of 0.4365. Nei's genetic diversity (h) and Shannon's information index (I) ranged from 0.0112 to 0.4997 and 0.0348 to 0.6928 respectively with average Nei's genetic diversity of 0.2597. The gene flow (Nm) 0.9702 and the genetic diversity of 0.2586 at species level demonstrated overall high level of genetic diversity. Cladistics analysis using DARwin and Bayesian cluster analysis using STRUCTURE placed 91 individuals into two main clusters or original genetic stocks (K = 2) which show little or no association with the geographic origin. The lack of clear assignment of individuals to geographical regions of sampling and consideration of different populations in the same genetic cluster suggests a recent common evolutionary history.
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Original Article

Genetic diversity and population structure of *Melia azedarach* L. growing in Indian Thar Desert.

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Abstract

Melia azedarach L is ecologically imperative species growing in Thar Desert. Biological effects such as antiviral, anthelminthic, antibacterial, etc. makes it important, yet a little is known about its genetic diversity and structure. In this study, we employed 30 RAPD primers for DNA profiling of 91 individuals representing 14 geographically isolated populations. A total of 104 bands were scored with an average of 3.5 bands per primer. The PIC ranged from 0.1898 to 0.5000 with an average of 0.4365. Nei's genetic diversity (h) and Shannon's information index (I) ranged from 0.0112 to 0.4997 and 0.0348 to 0.6928 respectively with average Nei's genetic diversity of 0.2597. The gene flow (Nm) 0.9702 and the genetic diversity of 0.2586 at species level demonstrated overall high level of genetic diversity. Cladistics analysis using DARwin and Bayesian cluster analysis using STRUCTURE placed 91 individuals into two main clusters or original genetic stocks (K = 2) which show little or no association with the geographic origin. The lack of clear assignment of individuals to geographical regions of sampling and consideration of different populations in the same genetic cluster suggests a recent common evolutionary history.

Key message: *M. azedarach* is a good reforestation tree because of its fast growth and drought hardy nature. This study adds a foundation for more precise inference about the biogeography and management in the reforestation projects in the Thar Desert.

Keywords: Genetic diversity, Gene flow, Thar Desert, Dendrogram.

Introduction

Melia azedarach L, a forest tree belongs to family Meliaceae, known as bead tree, Persian lilac, Paradise tree, Bakain, is native to north western India and China. It is grown widely in the world due to its high adaptability to a wide range of climatic conditions which naturalized it in other countries (Olmos et al. 2002; Yulianti et al. 2011). It is an important fast growing forest tree (Scocchi et al. 2004) which grows well in deep and sandy clay soils with good drainage having an alkaline pH of around 5.5-6.5. It grows at hills with elevation of 700-1400m above sea level and with a rainfall of 600-2000 mm/year and in warmer conditions (Yulianti et al. 2011). It is considered important for reforestation programms (Scocchi et al. 2004) and commercial purposes because of its good wood characteristics (Scocchi et al. 2004) and several biological effects including antifeedant (De Nardo et al. 1997), anthelminthic (Hördegen et al. 2003), antiviral (Andrei et al., 2003), antibacterial (Khan et al. 2001), and antiparasitic (Szewczuk et al. 2003) etc.

Due to restricted or limited mobility of plants, genetic structure refers to spatial structure or actual geographical distribution of plants which results from different environmental or ecological traits along with migration, dispersion, mutation, natural selection, genetic drift etc. Small, localized populations are more susceptible to genetic drift and limited gene flow as compared to widely distributed species resulting in an increased genetic diversity between populations and decreased genetic diversity within populations (Loveless et al. 1984). Limited gene flow between populations of plant species cause genetic differentiation due to increased geographic distance among populations or distant spatial pattern of populations. Alteration and fragmentation of many habitats lead to increased geographic distance which may be the cause of low level of gene flow and high genetic diversity among populations (Fischer et al. 2000).

Genetic variation in a species will allow itself to respond to environmental stress, adapt, evolve and survive for a long term. Severe environmental stress play an important role in revealing evolutionary history of forest trees (Sheng et al. 2005). Information about genetic structure of forest species of desert region not only provides information regarding significance of evolutionary forces such as gene flow, genetic drift, mutation and migration under extreme environmental conditions but also provides basic information for designing plans related to restoration and rational exploitation (Doligez et al. 1997; Fischer et al. 2000).

As many forest trees are of economic importance and their breeding is slow, so it is desirable to detect functionally important regions in the genome. Genetic diversity can be studied with a number of molecular markers, but RAPD is preferred because it is relatively easy and cheap method for detecting polymorphism within short duration and requires limited amount of DNA (Hadrys et al. 1992; Welsh et al. 1990).

In the present study RAPD technique was adopted to examine the genetic structure and diversity within and between 14 populations of *M. azedarach* in Thar desert.

Materials and Methods

Plant material

A total of 14 populations consisting of 91 individuals of *M. azedarach* have been collected from 14 different geographically isolated regions of Thar Desert. 6 populations were collected from Bathinda region of Punjab

and 8 populations were collected from Jodhpur, Jaisalmer and Raisinghnagar regions of Rajasthan. Within each region populations were isolated from each other at a distance of at least 50-60 Km and their size varied from 5-10 plants. Complete information regarding sample collection and location is given in Fig 1. and Table 1. Fresh leaves of each selected plant were collected and kept inside multiple layers of blotting sheets for transportation to the laboratory.

DNA isolation and RAPD analysis

Total DNA was extracted from leaf tissue by the CTAB method (Doyle 1990) with some modifications. The quality and concentration of the extracted DNA were estimated on 0.8% agarose gel and Nano Drop 2000 Spectrophotometer.

Genomic DNA was analysed using standard 10 mer oligonucleotide RAPD molecular markers by Williams et al method with some modifications (Williams et al. 1990). We standardized the DNA amplification conditions by assessing DNA concentration, primer concentration, different bands and concentrations of Taq polymerase as well as temperatures for PCR amplifications. Reactions were carried out in a final volume of 20ul containing 25mM $MgCl_2$ solution, 2.5mM dNTPs,10 picomoles primer, 25ng/ul of genomic DNA, 1.5 U of Taq polymerase (Banglore Genei). Amplifications were performed in a thermal cycler (Applied Biosystems Veriti 96 well) programmed for initial denaturation cycle of 5 min at 94°C followed by 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 37°C and extension for 2 min at 72°C with a final extension of 7 min at 72°C.

PCR amplified products mixed with 6X loading dye were electrophoresed on 2% agarose gel prepared in 1X TAE, stained with EtBr at 55V for 90 minutes; then visualized and photographed with the BIORAD Gel Doc X - imager.

Data analysis

Each fragment amplified using RAPD was treated as a binary unit character and scored 0 and 1 for absent and present bands respectively. Only intense and clearly visible bands were scored. Polymorphism information content (PIC) and percentage polymorphism were calculated for each primer which gives estimate about discrimination ability of a marker by considering number of alleles at a locus along with their relative frequencies (Table 2).

Genetic diversity was estimated using POPGENE software version 1.31 (Yeh et al. 1999) to determine different parameters which include number of polymorphic loci and their percentage (Table 3), observed number of loci, effective number of alleles ($n_e = 1/\sum p_i^2$, where p_i = frequency of the *i*th allele for the studied locus), Nei's genetic diversity ($h= \sum h_k/r$, where h_k = the value of h for the $_k$th locus and r = number of alleles studied) (Nei 1973), Shannon's information index (Table 1) and dendrograms. The phylogenetic relationship among populations was generated using software DARwin.

STRUCTURE (version 2.3) (Pritchard et al. 2000) was used to perform a Bayesian analysis to identify hidden population structure by delineating individuals into genetically distinguishable clusters on the basis of their genotypes at multiple loci. The optimal value of K was identified using both the adhoc procedure introduced by Pritchard et al (2000) (Fig 3.) and method developed by Evanno et al (2005) (Fig 4.). Population assignment test based on a Bayesian approach was conducted for all the samples using the software STRUCTURE for assigned number of populations of K=1 to 14 and with 3 replicates with a random start for each K value and 1000 runs. The number of distinct population clusters were determined using Δ K method (Evanno et al. 2005).

Results

Out of the 80 primers that were evaluated in 14 populations, 39 primers were polymorphic and finally 30 primers were chosen on the basis of generation of stable and reproducible bands in all the samples. A total of 104 polymorphic bands were generated with an average of 3.5 bands per primer and 0.4365 average polymorphism information content (PIC). The highest numbers of polymorphic bands were achieved with primers OPA07, OPB12, OPC15 and OPD02, while the most informative primer was OPA04 with PIC value of 0.5879 (Table 2). The number of polymorphic loci ranged from 33 (Kesrisinghpur) to 70 (New Pali road) within 14 populations with an average of 48. The percentages of polymorphic loci ranged from 31.73% (Kesrisinghpur) to 67.31% (New Pali Road) with an average of 46.22 (Table 3).

Eight parameters were used to assess the genetic variation within populations and they included number of loci or bands, number and percentage of polymorphic loci, actual or observed number of alleles (na), effective number of alleles (ne), Nei's genetic diversity (h), Shannon information index (I), expected or genetic diversity within population (intrapopulation diversity for subdivided populations). The highest number of observed alleles (na=1.6731) were present in New Pali Road population. The highest effective number of alleles (ne=1.4171), Nei's genetic diversity (h=2.375), Shannon's information index (I=0.3500) was found in Raisinghnagar population (Table 1).

Five parameters were calculated to investigate the genetic variation among populations and these included expected heterozygosity (H_T) or total genetic diversity for all populations, genetic differentiation between subpopulations relative to total genetic diversity ($G_{ST} = 1 – h_s/h_T$ where h_S= population diversity and h_T = total diversity), estimate of gene flow [Nm = 0.5(1 - Gst)/Gst], Nei's genetic distance and Nei's genetic identity (Supplementary Figure 1). The average genetic diversity was 0.1707 within population (Hs) and 0.2568 among populations or at species level (Ht). Average estimate of F_{ST} or G_{ST} across all loci were significantly different from zero (average G_{ST} = 0.3401) which indicated limited gene flow and genetic structuring amongst sampling locales (supplementary Table 1).

Dendrogrm was drawn by Dendrogram based on Neis's genetic distance method (UPGMA modified from Neighbor procedure of PHYLIP version 3.5) to visualize the relationships among 14 populations of *M. azedarach* L. (Figure 2). The results represented two clusters, one consisting of Gharsana and Anupgarh and the other consisting of the other 12 populations. Second cluster further consisted of two subclusters, one of which included Bathinda, Kesrisinghpur, Padampur and Jaisalmer and the other subcluster consisted of Hanumangarh, Dabbali, Ganganagar, NewPali road, Raisinghnagar, Karanpur, Mahajan and Lunkaransar.

According to Nei's unbiased measures of genetic distance (1978) data, the highest genetic distance pairs were between New Pali road and Bathinda (0.2057), New Pali road and Gharsana (0.1938), new Pali road and Kesrisinghpur (0.1923), New Pali road and Anupgarh (0.1749) and New Pali road and Jaisalmer (0.1664). Therefore, it indicates New Pali road population to be the most differentiated of all the populations. Data of Nei's (1978) Unbiased measures of genetic identity validates the data of genetic distance and the highest genetic identity pairs were between Hanumangarh and Ganganagar (0.9682), Hanumangarh and Dabbali (0.9665), Ganganagar and Dabbali (0.9624), Ganganagar and Raisinghpur (0.9608) and Hanumangarh and Raisinghpur (0.9604). Therefore it indicates that Hanumangarh, Ganganagar, Dabbali and Raisinghpur are highly similar on genetic level. Hence, these populations were showing strong relationships between them (Supplementary Figure 1). Unrooted tree constructed using DARwin determines phylogenetic relationship between the individuals on the basis of genetic distance and indicated two clusters of original genetic stocks and one cluster consisting of individuals migrated from the two original genetic stocks (Fig 6.).

STRUCTURE version 2.3 based on Bayesian approach was applied to search for hidden population structure among genotypes of different populations of *M. azedarach.* The relative distinctiveness of sampled populations was determined by assigning individuals on the basis of genetically homogeneous groups rather than on geographical locations of sampling. The highest value of ΔK identified 2 clusters or genetic stocks that were present among 14 sampled populations. This grouping is consistent with UPGMA dendrogram. Graphical method used to detect true number of populations i.e. K indicated mostly the number of genetic stocks at K=2 considering ΔK distribution (Fig 4.). Out of 91 individuals, 47 individuals were observed in the admixed stock and 40 and 4 individuals were in the two original genetic stocks (Fig. 3). Of the 91individuals sampled, first smaller genetic cluster occupied 4.39% (4 individuals from 3 populations), second larger genetic cluster occupied 43.95% (40 individuals from 13 populations) and the admixed cluster occupied 51.65% (47 individuals from 13 populations) (Fig 5.).

Discussion

Genetic diversity, a basic source of biodiversity is the total number of traits leading to variation within or among populations of a species (Poczai et al.). Maintenance of genetic diversity and population distinctiveness plays an important role in biodiversity and conservation of species as it helps to understand the processes or factors involved in genetic variation in populations and species (Nongrum et al. 2012).

RAPD has been used to detect genetic diversity within and between populations of *Populus euphratica* (Saito et al. 2002), *Vitellaria paradoxa* (Fontaine et al. 2004), *Gliricidia sepium* (Dawson et al. 1995), *Mangifera indica* (Díaz-Matallana et al. 2009) and many others. High values of percentage polymorphism for all the primers indicated that the main cause for high genetic diversity may be geographic isolation which regulates the process of genetic diversity and variation. High level of genetic diversity due to geographic isolation in *Anethum graveolens* populations and RAPD was used to reveal its genetic structure (Suresh et al. 2013). Population wise highest value of percentage of polymorphic loci is of New Pali road population (67.31%) which is geographically distant from other populations and dendrogram agrees with this data. This suggested that New Pali road population was the most distant and differentiated population. Present investigation using RAPD marker revealed that in *M. azedarach* highest genetic variation was observed among populations (H_T = 0.2586)

as compared to within population (H_S = 0.1707). Till now investigations regarding genetic diversity revealed low levels of genetic diversity within populations in case of populations of geographically restricted plant species but high levels of genetic diversity within population was observed in case of out-crossing species (Torres et al. 2003; Wagner et al. 2011).When genetic diversity is classified into within and among population genetic diversities, selfing species exhibit low levels of genetic diversity within population, but a considerable high genetic diversity among populations (He et al. 2007).

Genetic diversity among the populations and geographic range are highly related (Warghat et al. 2012). Several factors like geographical isolation, population fragmentation, breeding system and genetic drifts may be responsible for high population differentiation (Hogbin et al. 1999; Zong et al. 2008). F_{ST} values ranging from 0 to 0.05 represent little or very little genetic diversity, from 0.05 to 0.15 represent considerable genetic diversity and that >0.25 represent very high genetic diversity. The results of average F_{ST} (0.3401) indicated very high genetic diversity among populations. If populations are small and isolated from one another, the genetic drift could be capable of influencing the genetic structure and increasing differentiation among populations (Ellstrand et al. 1993). High genetic diversity indicated limited gene flow among *M. azedarach* populations which agrees with estimate of gene flow (Nm) obtained with POPGENE (version 1.31). Nm represents historical average levels of gene flow. A G_{ST} derived average genetic flow (Average Nm = 0.9702) revealed that gene flow between populations of *M. azedarach* is restricted and it is below the level (Nm>4) required to counteract genetic drift (i.e. Nm>4 according to stepping stone model) (Slatkin 1993). But in some population pairs Nm value revealed high gene flow.

If Nm>1 (in an infinite island model) or Nm>4 (in a stepping stone model), the gene flow is quite sufficient to create genetic differentiation between populations balanced for migration and genetic drift. But according to infinite island model, if 0.5>Nm>1, genetic differentiation among populations is small but considerable in case of stepping stone model (Díaz-Matallana et al. 2009). In the present study average Nm = 0.9702 which indicates limited gene flow among populations and agrees with the average F_{ST} value (0.3401) because F_{ST} is inversely related to Nm, the effective migration rate. A high F_{ST} indicates that populations are genetically differentiated the result of a low migration of genotypes.

Maximum gene flow was found between population pairs Hanumangarh and Ganganagar (Nm = 5.9542) followed by Ganganagar and Raisinghnagar (Nm = 5.3863), Hanumangarh and Raisinghnagar (Nm = 5.2081), Hanumangarh and Dabbali (4.4825) and Dabbali and Ganaganagar (Nm = 4.2580) (Table 4). All the other population pairs had Nm values <4 which indicates low gene flow (according to stepping stone model). These values agree with results of Nei's unbiased genetic identity and distance. Some of these populations are geographically closer and some are quite distant. This indicates that geographically closer populations undergo high gene flow when migrant genes arriving by pollen or seed or human interference become established in new genets (Ayres et al. 1999). Pollen dispersal by wind and insects between populations may be affected by environmental conditions, geographic distances between populations and topographic conditions (Islam et al. 2012). In the present study high gene flow was observed between populations which were geographically distant from each other. This may be due to human interference. Long distance seed dispersal by humans has been observed as a source of gene flow among populations of Italian wild Cherry (*Prunus avium* L.) (De Rogatis et al. 2013).

Clustering of different populations of *M. azedarach* in dendrogram based on Nei's genetic distance was not completely in congruent with their geographic locations and so it indicates that genetic diversity is not corroborating with geographical diversity. The data strongly suggests that overall genetic diversity is high and gene flow is limited but in some population pairs G_{ST} value is very low that is low genetic diversity and high Nm value (Nm>4) that is high gene flow. Overall high genetic diversity may be due to geographic isolation, habitat fragmentation or mating system but high gene flow in some population pairs may be due to human interference, pollen or seed dispersal etc.

Cluster analysis in *M. azedarach* showed that genetic diversity based on morphological traits was not in accordance to geographical regions (Solouki et al. 2008b). There was no clear clustering pattern of geographically closer individuals. Same results were obtained in the case of *Anethum graveolens L.* (Suresh et al. 2013). These observations clearly indicated that the association between genetic similarity and geographical distance was less significant. These genetic differences among populations may be due to selection, adaptation, migration, genetic drift and method of pollination. The other factor of importance is also vitally related to the environment and human interference (Solouki et al. 2008a). Dendrogram and cluster analysis showed that some landraces with same geographical area were clustered into different groups and no preferred relationship existed between geographical distribution and the dendrogram. Investigations related to cluster analysis have found clusters including trees from different populations which indicated that plants can be more similar to those from other populations than to those of their own population in case of *Ilex paraguariensis* (Gauer et al. 2000).

Both of the original genetic stocks consisted of individuals of populations from different geographical locations i.e. the two genetic clusters identified by STRUCTURE were not according to the geographical locations of sampling. This represented that a high level of gene flow has occurred. Structure infers the highest likelihood of both the individual clusters and the admixture of genotypes using allele frequency and linkage disequilibrium information from dataset directly. The Bayesian structure analysis, dendrogram and unrooted tree (Fig 6.) did not group. 14 *M. azedarach* populations may have originated from two ancestral gene pools and the presence of admixed cluster or stock suggested the occurrence of gene flow. None of the clusters determined by STRUCTURE either original or the admixed one consisted of specified full populations which indicated high gene flow.

Gene flow at the species level was limited due to wide geographical distribution or restricted seed and pollen dispersal. Gene flow and life history traits such as population size change, habitat fragmentation, bottlenecks etc. have been considered as the main determinants of population genetic structure. Therefore different populations considered in the same genetic clusters, suggests recent common ancestry to be the important factor rather than other events leading to gene flow.

Conclusion

This study has added significant knowledge and understanding to the genetic diversity and structure of *M. Azedarach* between and within the populations. Presence of high level of genetic diversity within and among the populations, Nei's genetic diversity, Shannon's information index and total gene diversity resulted in insignificant correlation between geographic distance and genetic diversity. The lack of clear assignment of

individuals to geographical regions of sampling and consideration of different populations in the same genetic cluster suggests a recent common evolutionary history and recent divergence.

Authors' contributions

NP, KA and GS collected samples and isolated DNA. NP and KA carried out DNA profiling and statistical analysis. RGS helped in drafting the manuscript. PB conceived the study, participated in designing, coordination, data analysis, interpretation, reviewed and improved the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figure

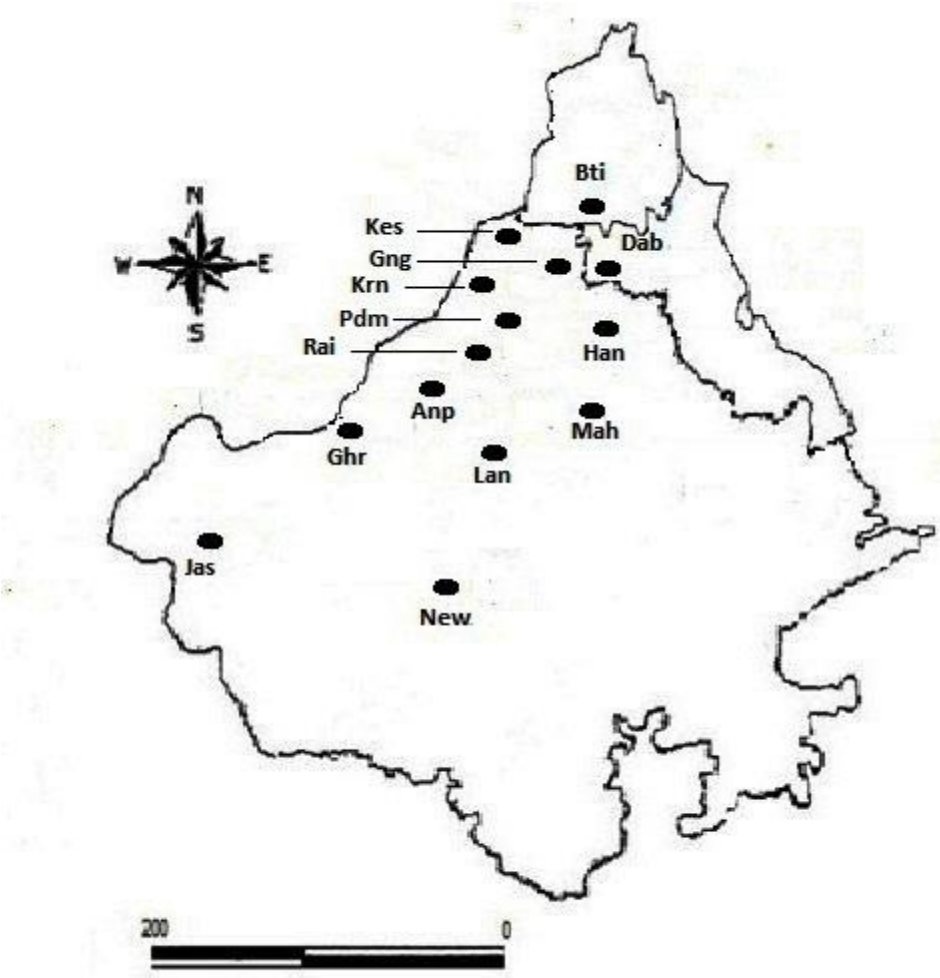

Fig 1: Sites of sample collection in North-western part (Thar Desert) of India.

Figure

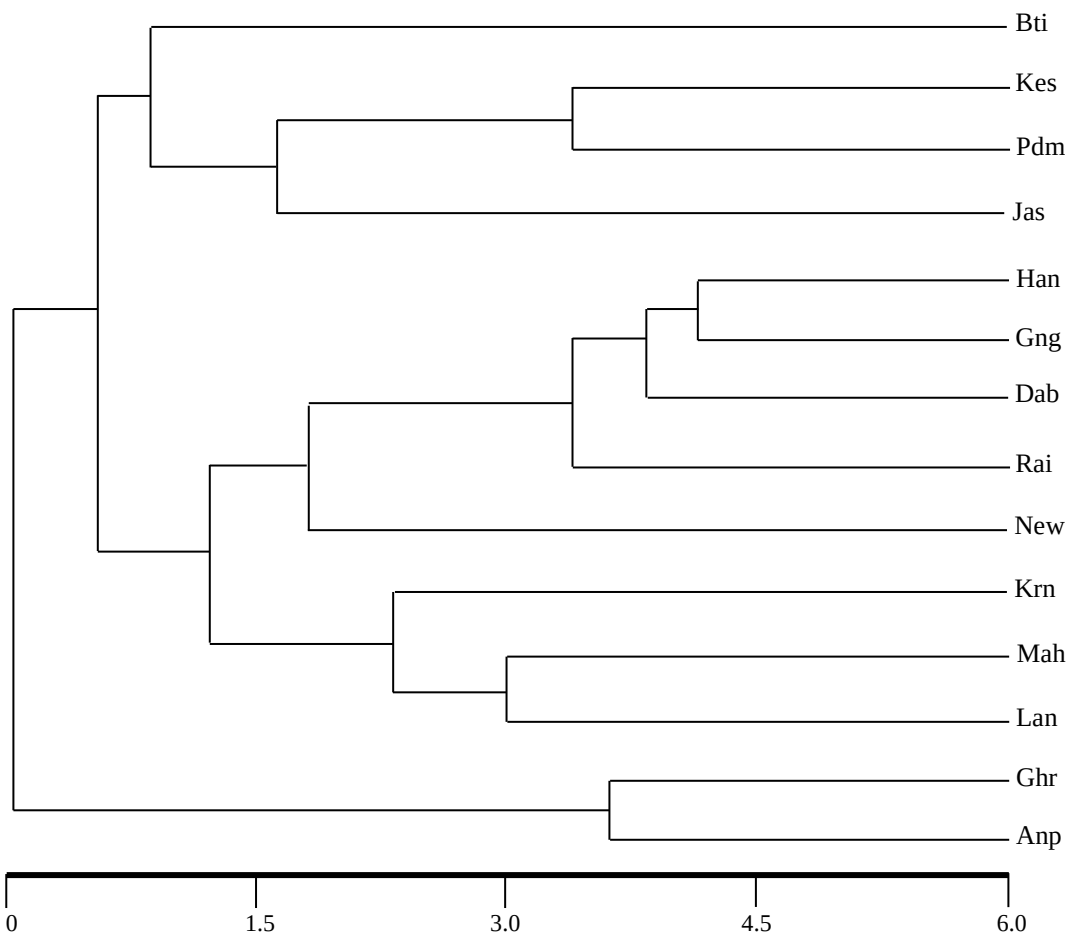

Fig 2. Dendrogram based on Nei's (1978) genetic distance for 14 popuations of *Melia azedarach L*.

Figure

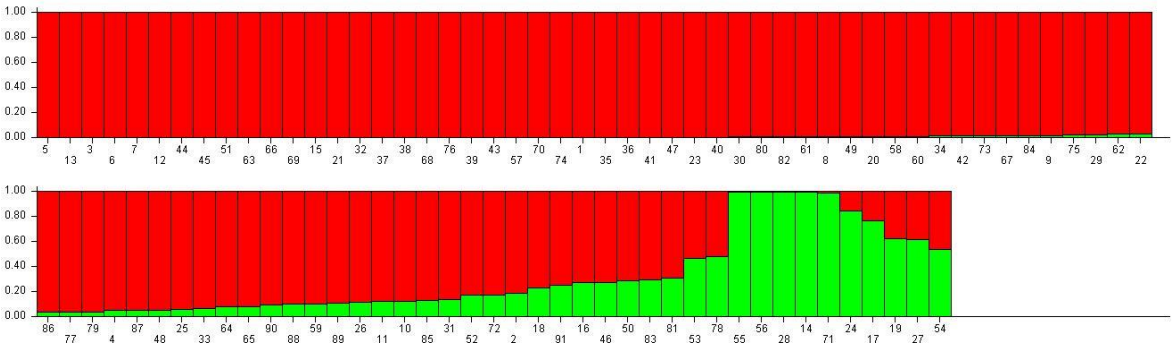

Fig 3. Structure plot with membership coefficient of individuals on y-axis. Assignment of all 91 individuals into two clusters. Membership coefficient of an individual for a subgroup represents fraction of its genome that has ancestry in subgroup. Two clusters inferred by Structure in combined clustering of all individuals. Each colour represents different genetic stock.

Figure

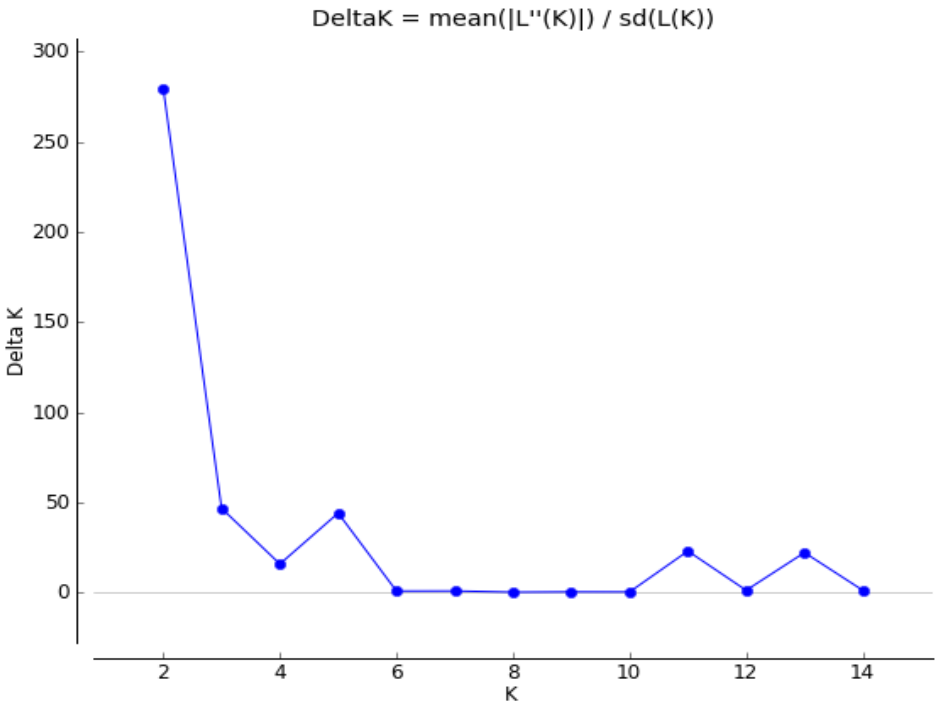

Fig 4. Most appropriate value of K from a range of 1-14 populations using the second order statistics (Δ K) given by Evanno et al. (2005)

Figure

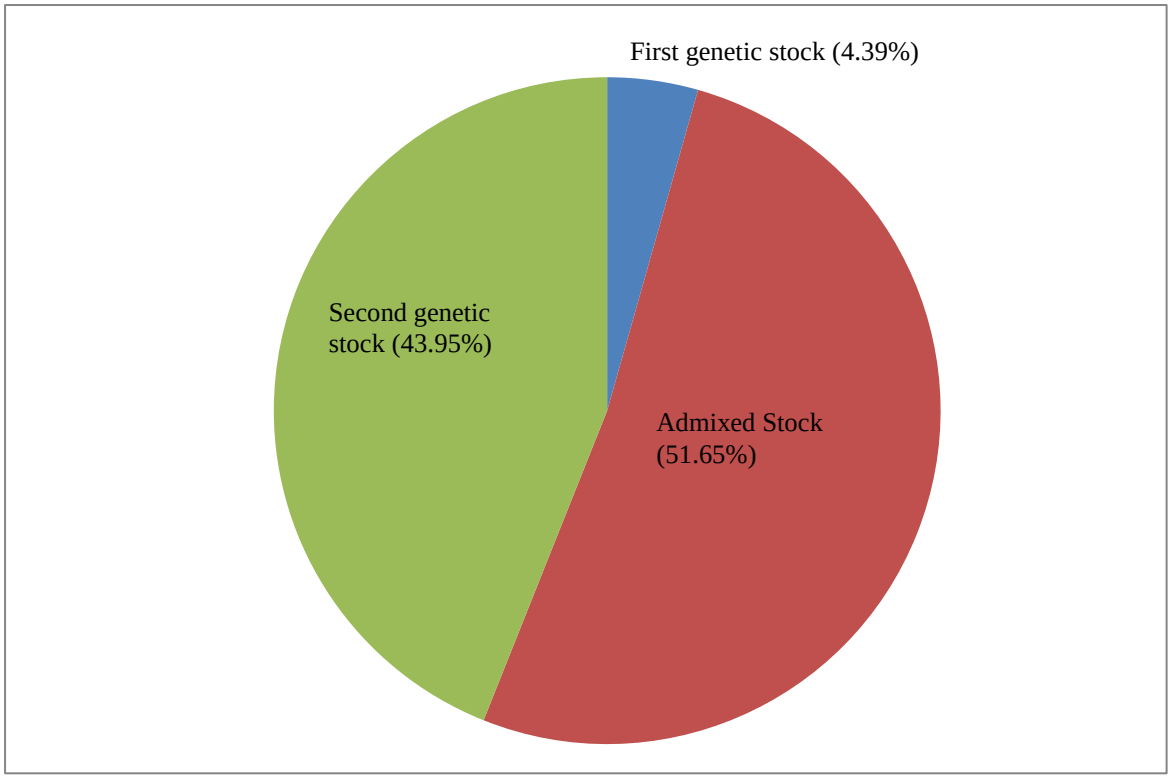

Fig. 5. Pie chart representing two original and one admixed genetic stock revealed by software STRUCTURE version 2.3.

Figure

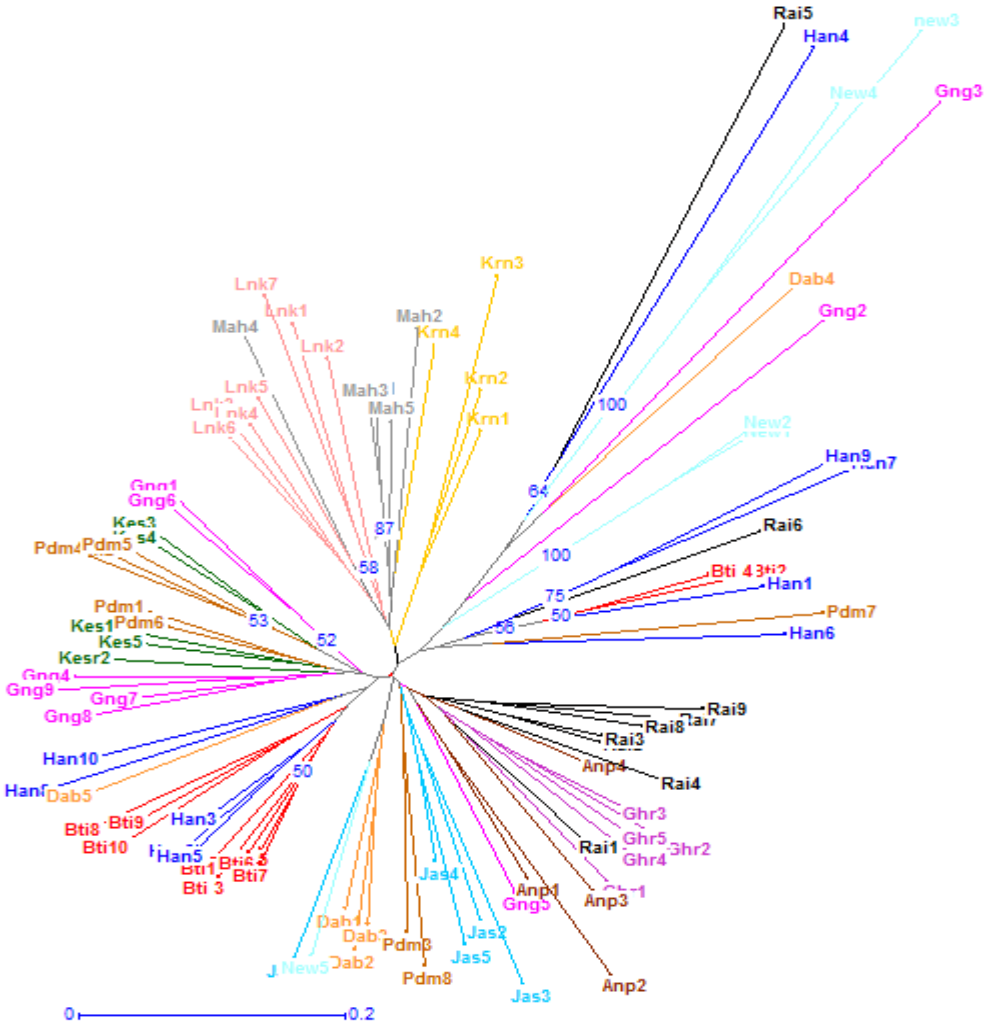

Fig 6. Unrooted tree construted by DARwin. Each coloured branch represents one individual collected from corresponding inferred population.

Table

Table1: *Melia azedarach* populations from Thar Desert and their genetic variability detected by RAPD analysis.

Population	Sample Size	Longitude (E)	Latitude (N)	Na	Ne	h	I
Bathinda (Bti)	10	74°47’29.52”E	30°08’26.96”N	1.4519	1.2788	0.1584	0.2356
Hanumangarh (Ham)	10	74°17’32.04”E	29°36’48.33”N	1.5962	1.3621	0.2094	0.3119
Dabbali (Dab)	5	74°38’46.09”E	29°55’28.72”N	1.3942	1.2984	0.1631	0.2358
Ganga nagar (Gng)	9	73°51’32.55”E	29°55’12.69”N	1.6346	1.4047	0.2286	0.3384
Kesrisinghpur (Kes)	5	73°37’16.18”E	29°56’51.68”N	1.3173	1.1934	0.1125	0.1684
Padampur (Pdm)	8	73°37’34.83”E	29°42’25.86”N	1.5096	1.3318	0.1881	0.2779
Jaisalmer (Jas)	5	70°21’48.26”E	26°52’23.50”N	1.4135	1.3021	0.1680	0.2443
New Pali Road (New)	5	72°59’57.12”E	26°13’17.32”N	1.6731	1.3609	0.2222	0.3398
Gharsana (Ghr)	5	73°04’43.72”E	29°01’18.57”N	1.3462	1.2158	0.1237	0.1845
Anupgarh (Anp)	4	73°12’34.18”E	29°11’21.07”N	1.3558	1.2757	0.1489	0.2148
Raisinghnagar (Rai)	9	73°26’56.75”E	29°32’09.01”N	1.6346	1.4171	0.2375	0.3500
Karanpur (Krn)	4	76°58’14.85”E	26°10’55.44”N	1.3750	1.2479	0.1405	0.2079
Mahajan (Mah)	5	73°50’12.67”E	28°47’16.13”N	1.3462	1.2479	0.1384	0.2019
Lunkaransar (Lnk)	7	73°45’18.23”E	28°30’01.75”N	1.4231	1.2533	0.1499	0.2247

na = Observed number of alleles

ne = Effective number of alleles

h = Nei’s gene diversity

I = Shannon’s Information index

Table

Table 2: Data of RAPD primers used in the present study and the extent of polymorphism.

S.No.	Primer Name	Primer Sequence(5'-3')	Amplified Bands	Polymorphic Bands	% Polymorphism	PIC
1.	OPA 01	CAGGCCCTTC	1	1	100	0.4680
2.	OPA 02	TGCCGAGCTG	4	4	100	0.4882
3.	OPA 04	AATCGGGCTG	4	4	100	0.5000
4.	OPA 05	AGGGGTCTTG	4	4	100	0.4996
5.	OPA 07	GAAACGGGTG	5	5	100	0.4082
6.	OPA 09	GGGTAACGCC	4	4	100	0.4652
7.	OPA 10	GTGATCGCAG	4	4	100	0.4933
8.	OPA 11	CAATCGCCGT	4	4	100	0.2753
9.	OPA 12	TCGGCGATAG	3	3	100	0.4232
10.	OPA 15	TTCCGAACCC	4	4	100	0.2789
11.	OPA 17	GACCGCTTGT	3	3	100	0.2898
12.	OPA 18	AGGTGACCGT	3	3	100	0.1898
13.	OPB 07	GGTGACGCAG	3	3	100	0.4419
14.	OPB 08	GTCCACACGG	4	4	100	0.2615
15.	OPB 10	CTGCTGGGAC	2	2	100	0.4990
16.	OPB 11	GTAGACCCGT	2	2	100	0.4970
17.	OPB 12	CCTTGACGCA	5	5	100	0.4374
18.	OPB 14	TCCGCTCTGG	3	3	100	0.4994
19.	OPB 15	GGAGGGTGTT	2	2	100	0.4707
20.	OPB 17	AGGGAACGAG	3	3	100	0.4991
21.	OPB 18	CCACAGCAGT	4	4	100	0.4815
22.	OPC 02	GTGAGGCGTC	3	3	100	0.4394
23.	OPC 08	TGGACCGGTG	3	3	100	0.4602
24.	OPC 11	AAAGCTGCGG	2	2	100	0.4864
25.	OPC 12	TGTCATCCCC	3	3	100	0.4875
26.	OPC 15	GACGGATCAG	5	5	100	0.3666
27.	OPC 18	CACACTCCAG	4	4	100	0.4623
28.	OPD 02	GGACCCAACC	5	5	100	0.4585
29.	OPD 03	GTCGCCGTCA	4	4	100	0.5000
30.	OPD 04	TCTGGTGAGG	4	4	100	0.4815

PIC = Polymorphism Information Content

Table

Table 3: Different populations with Number of polymorphic loci and percentage polymorphic loci.

Population	**Number of polymorphic loci**	**Percentage of polymorphic loci**
Bathinda	47	45.19 %
Hanumangarh	62	59.62 %
Dabbali	41	39.42 %
Ganga nagar	66	63.46 %
Kesrisinghpur	33	31.73 %
Padampur	53	50.96 %
Jaisalmer	43	41.35 %
New Pali Road	70	67.31 %
Gharsana	36	34.62 %
Anupgarh	37	35.58 %
Raisinghnagar	66	63.46 %
Karanpur	39	37.50 %
Mahajan	36	34.62 %
lunkaransar	44	42.31 %

Table

Table 4. Populations with maximum gene flow.

Population pairs	H_T	H_S	G_{ST}	Nm
Hanumangarh & Ganganagar	0.2374	0.2190	0.0775	5.9542
Ganganagar & Raisinghnagar	0.2546	0.2330	0.0849	5.3863
Hanumangarh & Raisinghnagar	0.2449	0.2234	0.0876	5.2081
Hanumangarh & Dabbali	0.2070	0.1862	0.1004	4.4825
Dabbali & Ganganagar	0.2188	0.1958	0.1051	4.2580

H_T = Total genetic diversity

H_S = Subpopulation genetic diversity

G_{ST} = Genetic differentiation between subpopulations relative to the genetic diversity of the total population.

Nm = Estimate of gene flow from G_{ST}

	Bathinda	Hanumangarh	Dabbali	Ganga nagar	Kesrisinghpur	Padampur	Jaisalmer	New Pali Road	Gharsana	Anupgarh	Raisinghnagar	Karanpur	Mahajan	Lunkaransar
Bathinda	****	0.9392	0.9162	0.9024	0.8990	0.9187	0.8764	0.8141	0.8861	0.8901	0.9181	0.8691	0.8751	0.8864
Hnumangarh	0.0627	****	0.9665	0.9682	0.8671	0.9124	0.8993	0.9249	0.8962	0.8919	0.9604	0.9057	0.8798	0.9119
Dabbali	0.0875	0.0340	****	0.9624	0.9002	0.9179	0.9042	0.9011	0.9023	0.9161	0.9477	0.8941	0.9126	0.8999
Ganga nagar	0.1027	0.0323	0.0383	****	0.9279	0.9371	0.9075	0.9486	0.8884	0.9007	0.9608	0.9314	0.9217	0.9252
Kesrisinghpur	0.1065	0.1426	0.1052	0.0748	****	0.9506	0.9042	0.8251	0.8607	0.8693	0.8897	0.8934	0.9061	0.8744
Padampur	0.0848	0.0917	0.0856	0.0650	0.0507	****	0.9201	0.8634	0.8926	0.9080	0.9414	0.9045	0.9064	0.9090
Jaisalmer	0.1320	0.1062	0.1007	0.0970	0.1007	0.0833	****	0.8467	0.8763	0.9061	0.9143	0.8903	0.8981	0.8939
New Pali Road	0.2057	0.0781	0.1042	0.0527	0.1923	0.1469	0.1664	****	0.8238	0.8395	0.9152	0.9092	0.8780	0.8903
Gharsana	0.1209	0.1096	0.1028	0.1184	0.1500	0.1136	0.1321	0.1938	****	0.9584	0.9369	0.8773	0.8561	0.8604
Anupgarh	0.1164	0.1144	0.0877	0.1046	0.1401	0.0965	0.0986	0.1749	0.0424	****	0.9437	0.8697	0.8832	0.8711
Raisinghnagar	0.0855	0.0404	0.0537	0.0400	0.1169	0.0604	0.0896	0.0886	0.0652	0.0579	****	0.9205	0.9164	0.9272
Karanpur	0.1403	0.0991	0.1119	0.0711	0.1128	0.1004	0.1162	0.0952	0.1309	0.1396	0.0828	****	0.9343	0.9305
Mahajan	0.1334	0.1281	0.0914	0.0815	0.0986	0.0982	0.1074	0.1301	0.1554	0.1242	0.0874	0.0680	****	0.9465
Lunkaransar	0.1206	0.0922	0.1055	0.0778	0.1342	0.0954	0.1121	0.1162	0.1504	0.1380	0.0756	0.0721	0.0550	****

Figure 1. Nei's unbiased measures of genetic identity and genetic distance (1978). Nei's genetic identity (above diagonal) and genetic distance (below diagonal). (POPGENE version 1.31).

Table 1. Nei's Genetic Variation Analysis

Locus	Sample Size	H_t	H_s	Gst	Nm
OPA01-1	91	0.5000	0.3276	0.3448	0.9499
OPA01-2	91	0.3031	0.1855	0.3879	0.7890
OPA01-3	91	0.4989	0.3040	0.3906	0.7801
OPA01-4	91	0.4897	0.2547	0.4800	0.5418
OPA02-1	91	0.1019	0.0918	0.0993	4.5344
OPA02-2	91	0.2145	0.1721	0.1978	2.0282
OPA02-3	91	0.4807	0.3601	0.2509	1.4929
OPA02-4	91	0.4479	0.3317	0.2594	1.4272
OPA04-1	91	0.4952	0.4038	0.1846	2.2084
OPA04-2	91	0.4821	0.3826	0.2063	1.9234
OPA04-3	91	0.1734	0.1512	0.1278	3.4130
OPA04-4	91	0.4872	0.3247	0.3335	0.9991
OPA05-1	91	0.4877	0.3298	0.3238	1.0439
OPA05-2	91	0.5000	0.3359	0.3282	1.0235
OPA05-3	91	0.4969	0.0920	0.8148	0.1136
OPA05-4	91	0.0317	0.0249	0.2127	1.8504
OPA07-1	91	0.3484	0.0935	0.7317	0.1833
OPA07-2	91	0.0317	0.0249	0.2127	1.8504
OPA07-3	91	0.1702	0.1409	0.1719	2.4086
OPA07-4	91	0.4800	0.3191	0.3352	0.9918
OPA09-1	91	0.4954	0.2637	0.4677	0.5691
OPA09-2	91	0.0500	0.0415	0.1700	2.4409
OPA09-3	91	0.0230	0.0212	0.0779	5.9225
OPA09-4	91	0.4999	0.3851	0.2297	1.6772
OPA10-1	91	0.3019	0.2463	0.1841	2.2160
OPA10-2	91	0.4935	0.3645	0.2613	1.4137
OPA10-3	91	0.0081	0.0077	0.0533	8.8767
OPA10-4	91	0.0150	0.0135	0.0988	4.5619
OPA11-1	91	0.4918	0.3922	0.2024	1.9698
OPA11-2	91	0.0261	0.0235	0.0992	4.5424
OPA11-3	91	0.2145	0.1744	0.1870	2.1745
OPA11-4	91	0.2683	0.1994	0.2566	1.4486
OPA12-1	91	0.4585	0.1830	0.6009	0.3320
OPA12-2	91	0.1721	0.1523	0.1151	3.8427
OPA12-3	91	0.3375	0.2857	0.1533	2.7611
OPA12-4	91	0.0485	0.0447	0.0776	5.9412
OPA15-1	91	0.0847	0.0717	0.1530	2.7670
OPA15-2	91	0.4303	0.3268	0.2405	1.5786
OPA15-3	91	0.0073	0.0070	0.0478	9.9544
OPA15-4	91	0.0531	0.0454	0.1456	2.9338
OPA17-1	91	0.0403	0.0370	0.0826	5.5513
OPA17-2	91	0.1168	0.1071	0.0833	5.4991
OPA17-3	91	0.1478	0.1244	0.1582	2.6604
OPA17-4	91	0.0956	0.0851	0.1099	4.0486
OPA18-1	91	0.0500	0.0415	0.1700	2.4409
OPA18-2	91	0.2930	0.1455	0.5034	0.4933
OPA18-3	91	0.3273	0.2831	0.1351	3.2019
OPA18-4	91	0.1431	0.1068	0.2539	1.4691
OPB08-1	91	0.4716	0.3178	0.3261	1.0333
OPB08-2	91	0.4997	0.3488	0.3020	1.1558
OPB08-3	91	0.1524	0.0195	0.8719	0.0735
OPB08-4	91	0.0317	0.0249	0.2127	1.8504
OPB11-1	91	0.1517	0.1306	0.1394	3.0878
OPB11-2	91	0.4940	0.2651	0.4635	0.5789

OPB11-3	91	0.4491	0.2357	0.4752	0.5521
OPB11-4	91	0.2473	0.1894	0.2340	1.6367
OPB12-1	91	0.5000	0.2628	0.4743	0.5542
OPB12-2	91	0.3949	0.2121	0.4628	0.5803
OPB12-3	91	0.0105	0.0098	0.0692	6.7204
OPB15-1	91	0.4986	0.4002	0.1973	2.0341
OPB15-2	91	0.1909	0.1553	0.1865	2.1813
OPB15-3	91	0.1559	0.1288	0.1736	2.3809
OPB17-1	91	0.4865	0.3229	0.3364	0.9864
OPB17-2	91	0.4724	0.3830	0.1894	2.1403
OPB17-3	91	0.4808	0.2756	0.4269	0.6713
OPB18-1	91	0.0317	0.0249	0.2127	1.8504
OPB18-2	91	0.0073	0.0070	0.0478	9.9544
OPB18-3	91	0.4075	0.2226	0.4538	0.6019
OPC12-1	91	0.0150	0.0135	0.0988	4.5619
OPC12-2	91	0.4765	0.3607	0.2431	1.5568
OPC12-3	91	0.3996	0.2452	0.3865	0.7937
OPC11-1	91	0.3874	0.2473	0.3617	0.8823
OPC11-2	91	0.0244	0.0233	0.0464	10.2715
OPC11-3	91	0.2694	0.1963	0.2714	1.3425
OPC18-1	91	0.0164	0.0156	0.0509	9.3323
OPC18-2	91	0.1465	0.0988	0.3254	1.0367
OPC18-3	91	0.1704	0.0944	0.4460	0.6212
OPC02-1	91	0.0317	0.0249	0.2127	1.8504
OPC02-2	91	0.2636	0.2157	0.1819	2.2490
OPC02-3	91	0.1050	0.0603	0.4261	0.6736
OPC08-1	91	0.0317	0.0249	0.2127	1.8504
OPC08-2	91	0.0167	0.0149	0.1106	4.0216
OPC08-3	91	0.3536	0.2009	0.4317	0.6582
OPC15-1	91	0.0230	0.0212	0.0779	5.9225
OPC15-2	91	0.4639	0.2863	0.3829	0.8059
OPC15-3	91	0.0670	0.0623	0.0706	6.5808
OPD02-1	91	0.0317	0.0249	0.2127	1.8504
OPD02-2	91	0.3280	0.2027	0.3822	0.8082
OPD02-3	91	0.4840	0.2723	0.4374	0.6431
OPD03-1	91	0.4141	0.2757	0.3342	0.9961
OPD03-2	91	0.0230	0.0212	0.0779	5.9225
OPD03-3	91	0.4996	0.1896	0.6204	0.3059
OPD04-1	91	0.0081	0.0077	0.0533	8.8767
OPD04-2	91	0.4838	0.2844	0.4122	0.7130
OPD04-3	91	0.4061	0.2558	0.3702	0.8506
OPB07-1	91	0.0167	0.0149	0.1106	4.0216
OPB07-2	91	0.1487	0.1022	0.3128	1.0983
OPB07-3	91	0.4320	0.2918	0.3246	1.0405
OPB14-1	91	0.0154	0.0147	0.0470	10.1322
OPB14-2	91	0.0618	0.0353	0.4290	0.6656
OPB14-3	91	0.0366	0.0306	0.1627	2.5728
OPB10-1	91	0.4578	0.2266	0.5050	0.4902
OPB10-2	91	0.4873	0.3120	0.3597	0.8902
OPB10-3	91	0.2496	0.2120	0.1506	2.8198
Mean	91	0.2586	0.1707	0.3401	0.9702

H_T = Total genetic diversity

H_S = Subpopulation genetic diversity

G_{ST} = Genetic differentiation between subpopulations relative to the genetic diversity of the total population.

Nm = Estimate of gene flow from G_{ST}