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Genetic diversity study of chinaberry plants with the use of molecular markers

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Abstract

Genetic variation in the natural growing populations appears due to challenging ecological and environmental conditions that leads to evolution. Molecular markers (RAPD & SSR) were applied to investigate ecological effects on the DNA of *Melia Azeadarch* L. Targeted plants were collected from various areas of Turkey and different locations of Pakistan. Genetic distances among all populations of Chinaberry plants were analyzed by generating dendogram which shows phylogenetic relationship among them. Different conservation strategies should be applied to conserve the genome of the naturally growing *M. azedarach* L. populations. Genomic DNA was extracted with the protocol followed by TM Plant genomic DNA Mini-prep Kit method. Total amount of DNA was quantified through Qubit® 2.0 Fluorometer. PCR based RAPD and SSR markers were applied for the study of genetic polymorphism among chinaberry DNA samples collected from five different locations of Turkey and three different areas of Pakistan. RAPD and SSR Molecular markers were applied which showed polymorphic bands. According to Jaccard's similarity index with RAPD markers highest diversity was noted between Edirne and Izmir while the highest similarity index was noted between the genome of Istanbul and Adana. With the applications of SSR markers highest similarity index was noted among genomes of Edirne and Kocaeli. For the conservation and use of plants the study of genetic diversity and its distribution is most important. That will support in determining what to conserve and where to conserve, and will helpful to understand of the taxonomy and origin of targeted plant species. In order to manage germplasm conservation, there is a need to understand the genetic diversity of the wild plants.

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Introduction

M. azedarach L. belongs to family *Meliaceae* commonly known as Chinaberry or Persian lilac, well grows in alkaline soil and waterlogged conditions (Tomar *et al.* 2003). Morphologically it attains 10 to 15 meters height. Chinaberry is native to India and China, now has been cultivated to other parts of the world and considered as highly invasive species in several tropical and subtropical areas. It prefers among cold climate and is a fast growing species; its food is used as component of agroforestry system Meena *et al.* (2018) and Tomer and Davies, (2003). Different parts of chinaberry such as leaves, stems, roots, flowers, seeds and fruit shave insecticidal properties Juan *et al.* (2000) and Ventura and Ito, (2000).

The DNA marker technique based on PCR is increasing with the passage of time for to study the genetic polymorphism among the plants. DNA fingerprinting approaches are available such as Amplified fragment length polymorphism (AFLP), Restriction fragment length polymorphism (RFLP), Simple sequence repeats (SSRs) and randomly amplified polymorphism (RAPD) Sasıkala & Kamakshamma, (2015). To understand the process of evolution, it is most important to study the genetic diversity and also the mutation and migration Doligez and Joly, (1997) and Fischer *et al.* (2000). The RAPD is cheap method, is extensively used for the study of genetic variability study in bacteria, fungi and plants Nybon & Bartish, (2000). RAPDs (Random Amplified Polymorphic DNA) are said to first molecular markers used to study the genetic diversity; it is one of the most popular molecular techniques having high speed low cost and simplicity.

Thus RAPD primers have been used in many crops providing rapid and appropriate assessment of genetic diversity among different genotypes (Govindaraj *et al.* (2015), Welsh and McClelland, (1990) and Williams *et al.* (1991). The duplication of unknown DNA sequences takes place with the use of RAPD markers on random bases and uses single short and random oligonucleotide primer Williams *et al.* (1990). SSR (Simple Sequence Repeats) and microsatellites are important markers applied for the genetic diversity study and population structure analysis. SSR markers serve as an important tool for ecological information and reproducibility Jaume *et al.* (2018). Genetic variation among species is the basis of evolution which plays an important role in their survival and adoptively in changed environmental conditions Thakur *et al.* (2016). The knowledge about genetic diversity within a population is most important to understand the conservation Catană *et al.* (2013). Present study was designed to use RAPD and SSR markers for genetic diversity analysis among two different populations of *M. azedarach* L. growing in Pakistan and Turkey. Genetic diversity within the population of *M. azedarach* L provides base line information to establish conservation strategies for the existing.

Material and method

Collection of Plant Materials

Leaves of *M. azedarach* L were collected from three locations in Sindh, Pakistan including Karachi (Labor Squire Landhi Karachi), Hyderabad (Hala Naka, Phuleli Canal, Hyderabad), KhairpurMirs (Setharja Village, Khairpur Mirs) and five locations in Turkey including Kocaeli (Izmit city near marmara sea, Kocaeli), Istanbul (Gülhane Park near Sultan Ahmad mosque, Istanbul), Izmir (Izmir University, Izmir), Edirne (Trakya University, Edirne) and Adana (Adana Science and technology University, Adana) as shown in fig-1. The population of these sites has diversity in the terms of vegetation structures, climate and soil as selected according to reported method of Pratt *et al.* (1977). The dust was removed from leaves with distilled water, wrapped in labeled aluminum foil and placed in liquid nitrogen container and stored at -40°C.

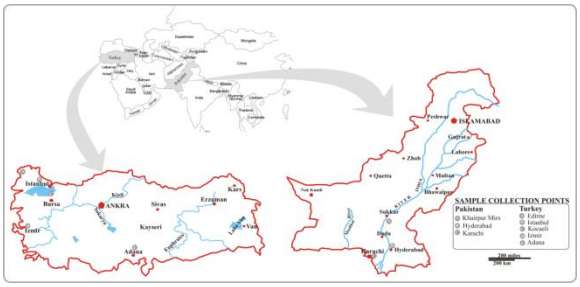

Fig. 1. Sample collection points of *Melia azedarach* L. from various areas of Turkey and Pakistan.

RAPD and SSR markers

Seventeen RAPD and four pairs of SSR markers were applied to study the genetic polymorphism as shown in table 1 and table 2. These markers were purchased from Eurofins MWG/Operon with respective dilutions of each primer.

Table 1. RAPD primers for the study of genetic polymorphism *Melia azedarach* L.

S. No.	Primer (RAPD)	Sequence	S. No	Primer (RAPD)	Sequence
1	OPX-13	5'-ACGGGAGCAA-3'	10	OPB-04	5'-GGACTGGAGT-3'
2	OPA-07	5'-GAAACGGGTG-3'	11	OPB-08	5'-GTCCACACGG-3'
3	OPA-10	5'-GTGATCGCAG-3'	12	OPB-09	5'-AGCAGCGCAC-3'
4	OPA-11	5'-ACCCGACCTG-3'	13	OPB-13	5'-GGGTCTCGGT-3'
5	OPA-13	5'-CAGCACCCAC-3'	14	OPAG-02	5'-CTGAGGTCCT-3'
6	OPA-15	5'-TTCCGAACCC-3'	15	OPAG-04	5'-GGAGCGTACT-3'
7	OPA-16	5'-AGCCAGCGAA-3'	16	OPQ-02	5'-TCTGTCGGTC-3'
8	OPA-17	5'-GACCGCTTGT-3'	17	OPQ-10	5'-TGTGCCCGAA-3'
9	OPB-01	5'-GTTTCGGTCC-3'			

Table 2. SSR markers for Genetic Polymorphism study of *M. azedarach* L.

SSR Marker		Primer Sequence 5' - 3'
NpCT_4	Left	5'-TGGTAACCAATCTGTGTGTGC-3'
	Right	5'-CGGTTCCTGGTTTCTTTTGG-3'
NpCT_5	Left	5'-GAAAGGAGGGTTTTCAAATCA-3'
	Right	5'-TCGGCCGAACACAATTTTA-3'
NpCT_34	Left	5'-ATTTGTGTGTGCGTGCTAGG-3'
	Right	5'-CGAGGAACTGAGACTCCTGAA-3'
NpCT_48	Left	5'-TCCCAGTTATTCAACGTAGGC-3'
	Right	5'-TCTTAATCATGGATTGCTTCACA-3'

DNA Extraction and Quantification

Leave samples of *M. azedarach* L stored at -40°C, for further experiments evaporated surface water, ground in liquid nitrogen and genomic DNA was extracted through a protocol and reagents followed by Sigma-Aldrich Gen Elute TM Plant genomic DNA mini-prep Kit. Total genomic DNA was quantified through Qubit® 2.0 Fluorometer. Firstly, reaction buffer was prepared by 1µL Qubit reagent with 199µL Qubit buffer. For DNA quantification, 1µL of DNA sample was mixed with 199µL of reaction buffer was mixed and quantified against standard. The standard was prepared by mixing 190µL of reaction buffer and 10µL of standard from kit.

PCR Amplification

For RAPD-PCR analysis, 25µl reaction mixture was prepared in autoclaved PCR tubes, comprised on 23µl PCR master mix, 1µl or RAPD marker and 1µl genomic DNA. The PCR programming was adjusted with an initial denaturation step one cycle of 15 minutes at 94°C, followed by step two 44 cycles at 94°C for 1minute denaturation, 36°C for 1 minute annealing and 72°C for 2 minutes extension and final step was adjusted for 5 minutes at 72°C PCR was allowed to ensure full extension of all amplified products Runo *et al.* (2004). The amplification of each sample was carried out in triplicate. For SSR-PCR, 25µl reaction mixtures was prepared in PCR tubes containing 22µl of PCR master mix, 1µl of forward and reverse SSR primers and finally 1µl genomic DNA was added separately. PCR programming for SSR primers was adjusted with an initial denaturation of step one cycle of 5 minutes at 94°C, followed by 40 cycles at 94°C for 1 minute denaturation, 54°C for one minute annealing and 72°C for 2 minutes extension of all amplified products Jones *et al.* (1997 and Wu and Huang, (2008). 100-bps DNA ladder (Gene-On) was resolved electrophoretically in 1% agarose gel contains 0.5µl Ethidium Bromide, and run at 50V for about 1 and ½ hours. RAPD and SSR banding patterns were visualized using (Bio-Rad) UV Gel documentation System.

Statically Analysis

The data was scored from the RAPD and SSR profiles through PAST version 3.08 were subjected to UPGMA cluster analysis to establish the relationship among the eight accessions selected from the different areas Hammer *et al.* (2001). Genetic similarity coefficient among the different localities of *M. azedarach* L was estimated through Jaccard's (1908) coefficient matrix. The polymorphism percentage was calculated by dividing the number of polymorphic bands over the total number of amplified bands and multiplied by hundred Qadir *et al.* (2015).

Results and discussion

For genetic polymorphism study 17 RAPD markers were used and their collective results showed the genetic similarity and diversity among the genome of plant samples collected from Pakistan and Turkey. Clear and reproducible PCR bands were produced

with almost all RAPD markers as shown in fig-2, these were further used for UPGMA dendogram and Jaccard´s similarity indexes. Total 490 clear RAPD-PCR bands were produced, out of these 266 bands were polymorphic with 54.28% polymorphism as shown in table-3. Similarly 224 monomorphic bands were produced. The highest genetic similarity 0.79167 (79%) was noted between the samples of Adana and Istanbul while the highest diversity index was noted 0.41(41%) between the samples of Kocaeli and Edirne as shown in table-4.

Table 3. Polymorphism in the genomes of eight *Melia azedarach* L plants using 17 RAPD primers with Primer length (10 bases) and 36°C Anneal temperatures.

Primer	Number of bands			% Polymorphism
	Total Bands	Polymorphic	Monomorphic	
OPA-07	18	2	16	11.11
OPA-10	24	24	0	100
OPA-11	43	11	32	25.58
OPA-13	31	15	16	48.38
OPA-15	27	27	0	100
OPA-16	29	21	8	72.41
OPA-17	26	2	24	7.69
OPB-01	18	10	8	55.55
OPB-04	45	21	24	46.66
OPB-08	20	20	0	100
OPB-09	32	0	32	0
OPB-13	26	10	16	38.46
OPAG-02	31	15	16	48.38
OPAG-04	36	28	8	77.77
OPQ-02	27	19	8	70.37
OPQ-10	27	3	24	11.11
OPX-13	48	40	8	83.33
	490	266	224	54.28%

Table 4. Similarity index using Jaccord's method with 17 RAPD markers.

	Adana	Istanbul	Kocaeli	Izmir	Edirne	Karachi	Hyderabad	Khairpur Mirs
Adana	1.000							
Istanbul	0.791	1. 000						
Kocaeli	0.517	0.592	1.000					
Izmir	0.72	0.75	0.592	1.000				
Edirne	0.464	0.428	0.413	0.481	1.000			
Karachi	0.461	0.541	0.461	0.541	0.666	1.000		
Hyderabad	0.666	0.695	0.538	0.695	0.608	0.619	1.000	
KhairpurMirs	0.583	0.608	0.52	0.681	0.521	0.454	0.619	1.000

Out of 490 PCR bands 266 bands were polymorphic average 15.76±10.88 were polymorphic while 224 bands with an average 14.11±10.40 were monomorphic. According to UPGMA dendogram as shown in fig.-3, the genomes of Istanbul and Adana has highest similarity while highest diversity (genetic distance) was noted between the samples of Edirne and Kocaeli. In eight *Meliaazedarach* L genotypes PCA analysis grouped Khairpur Mirs, Izmir, Istanbul, Hyderabad and Adana together, Karachi and Adana together while Kocaeli looks separate from all components.

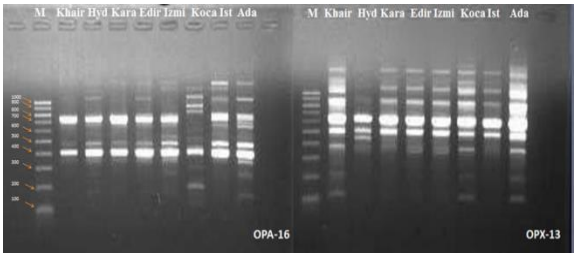

Fig. 2. Agarose Gel Electrophoresis of PCR products using OPX-13 and OPA-16 RAPD marker.

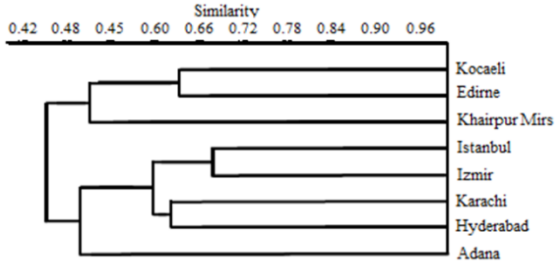

Fig. 3. UPGMA dendogram of the genome of eight different locations of Pakistan and Turkey 17 RAPD markers.

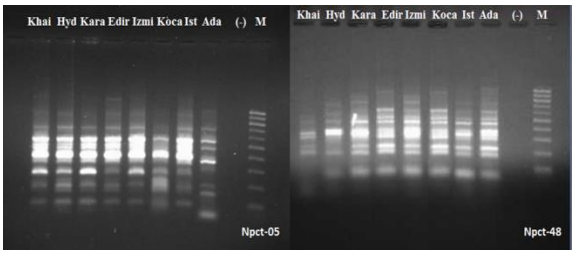

Fig. 4. PCR bands with SSR Npct-04, Npct-05, Npct-34 and Npct-48 forward and reverse markers.

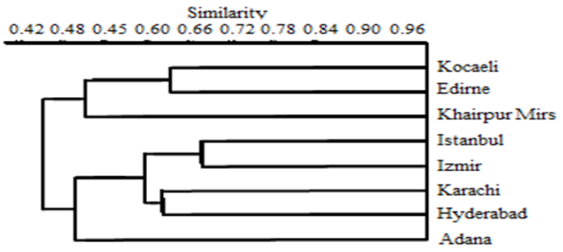

Fig. 5.UPGMA dendogram of the genome of eight different locations of Pakistan and Turkey 4 SSR markers.

Table 5. Similarity index among eight plant species with forward and reverse SSR markers (Npct-04, Npct-05, Npct-34 and Npct-48)

	Adana	Istanbul	Kocaeli	Izmir	Edirne	Karachi	Hyderabad	Khairpur Mirs
Adana	1. 000							
Istanbul	0.52	1.000						
Kocaeli	0.461	0.5	1.000					
Izmir	0.444	0.681	0.541	1.000				
Edirne	0.407	0.384	0.636	0.423	1.000			
Karachi	0.56	0.608	0.608	0.583	0.541	1.000		
Hyderabad	0.481	0.583	0.461	0.625	0.52	0.625	1.000	
Khairpur Mirs	0.363	0.4	0.473	0.380	0.555	0.526	0.304	1.000

The eight genotypes were also evaluated using four SSR markers (Npct-4, Npct-5, Npct-34 and Npct-48) forward and reverse. The analysis of SRR loci was done ranges from 0 (monomorphic) to 1. The PIC (Polymorphism Information Content) was ranged from 0.3 to 0.636. Clear and reproducible SSR-PCR bands were observed as shown in fig-4. The highest similarity index was observed (0.63) between the genomes of Edirne and Kocaeli while the lowest similarity with having the highest genetic distance was noted (0.3) between the samples of Hyderabad and Khairpur Mirs table-5. Among the Pakistani *Melia azedarach* samples the highest similarity index (0.62) was noted between the samples of Karachi and Hyderabad.

Beside the morphological traits for to evaluate the genetic diversity study plants has been influenced by environment. Therefore the molecular markers have been used to characterize the huge amount of genome in short time period for morphological study at genetic level Perry and McIntosh, (1991) and Masood *et al.* (2000). More than one marker systems like SSR and RAPD markers should be used for the study of high level of genetic resolution of the genome Tonk *et al.* (2014). Genetic diversity among the *Melia azedarach* L genotypes using RAPD and SSR markers which identifies relatively high number of polymorphic products Kizhakkayil. (2010) and Okon, (2011). In present study, samples from Pakistan and Turkey were investigated with the use of 17 RAPD markers and 4 SSR markers were used to evaluate the eight genotypes Rind *et al.* (2016). *M. azedarach* DNA samples belongs to Turkey having great genetic diversity with the samples from different locations in Sindh, Pakistan. The UPGMA dendogram based on the similarity matrix associated in to two major branches of clusters associations of Turkey and Pakistan. Among these samples, Adana and Karachi DNA samples have highest genetic diversity. Genetic diversity of samples showed higher possibility allowing them more easily to adapt under gradual environmental variations. According to PCA values genotypes divided in to three components that previously studied by Yagdi, (2012) and Qadir *et al.* (2015) both reported their study about the genotypes grouped in two and five PCA units.

For the assessment of the study of genetic diversity of phylogenic relationships within and among the plant populations RAPD can be considered as an essential tool. Many reports are available concerned with the determination of genetic variability with the use of RAPD markers Huenneke, (1991), Pither and Kellman, (2002) and Scocchi *et al.* (2004). Populations prefer to adopt their local climates and change their genome according to the change in climatic conditions. As indicated in previous reports that different climatic factor effects on DNA and lead to different type of living organisms Yang *et al.* (2018), Huang *et al.* (2005) and Jin and Liu, (2008).

Present study has been extended among the populations of *M. azedarach* genome belongs to eight different locations of Pakistan and Turkey. It has been evaluated that genetic diversity found among the plant species may be due to eco-geographical

differences and variable climatic conditions between both countries. Each PCR bands on the gel were analysed (0) absent and (1) present polymorphic bands of the individual lanes Kumar and Gurusubramanian (2011). The results in dendogram showed that main groupings could be associated with the geographical origin of genotypes Youn and Chung, (1998). Many factors can affect genetic diversity of plants like genetic drift; breeding system, distribution range and the way of seed disperse, in present study 55.33% polymorphism was noted through RAPD markers. SSRs are ideal genetic markers to detect the genetic diversity between and within the species of genes of all eukaryotic living organisms Farooq and Azam, (2002) andYang *et al.* (2018). It is reported that geno-toxic effects of environmental chemical pollutants, causes genetic damage that can exist longer period Gichner *et al.* (2008). Physical and chemical mutagenic factors due to different geographical localities cause irreversible DNA damages. UV ionizing radiations, high salinity and other chemical mutagenic agents showed effects on the genome Waterworth *et al.* (2011), Roy *et al.* (2013) and Yoshiyama *et al.* (2013). For the conservation of plant genetic resources two strategies like in situ and ex situ should be applied. In-situ implementation is particularly difficult due to anthropogenic influence Zawko *et al.* (2001). Ex situ conservation can be applied following various methods like seed storage, in vitro propagation, DNA storage, pollen storage, botanical gardens (Nybom, 2004). *M. azedarach* Lpopulations should be conserved because the mixed populations may give rise to out breeding depression, loss of adaptation or breakup of eco-adapted gene complexes (Toro and Caballero, 2005) which should be avoided during *M. azedarach* L conservation.

Conclusion

For the study of genetic polymorphism seventeen "17" RAPD markers were used highest genetic similarity index was noted between the samples of Adana and Istanbul. The eight "8" genotypes were evaluated with uniquely using four "4" SSR markers produced clear and reproducible PCR bands, highest similarity index was observed between the genome of Edirne and Kocaeli.

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