Genetic diversity in the candidate trees of *Madhuca indica* J. F. Gmel. (Mahua) revealed by inter-simple sequence repeats (ISSRs)

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Abstract

Madhuca indica provides livelihood to several tribal people in India, where the flowers are used for extraction of sweet juices having multiple applications. Certain trees have more value as judged by the tribal people mainly based on yield and quality performance of the trees, and these trees were selected for the genetic diversity analyses. Genetic diversity of 48 candidate Mahua trees from Etapalli, Dadagaon, and Jawhar, Maharashtra, India, was assessed using ISSR markers. Fourteen ISSR primers revealed a total of 132 polymorphic bands giving overall 92% polymorphism. Genetic diversity, in terms of expected number of alleles (N_e), the observed number of alleles (N_a), Nei's genetic diversity (H), and Shannon's information index (I) was 1.921, 1.333, 0.211, and 0.337, respectively, and suggested lower genetic diversity. Region wise analysis revealed higher genetic diversity for site Etapalli ($H = 0.206$) and lowest at Dhadgaon ($H = 0.140$). Etapalli area possesses higher forest cover than Dhadgaon and Jawhar. Additionally, in Dhadgaon and Jawhar *M. indica* trees are restricted to field bunds; both reasons might contribute to lower genetic diversity in these regions. The dendrogram and the principal coordinate analyses showed no region-specific clustering. The clustering patterns were supported by AMOVA where higher genetic variance was observed within trees and lower variance among regions. Long-distance dispersal and/or higher human interference might be responsible for low diversity and higher genetic variance within the candidate trees.

Keywords Genetic diversity · ISSR markers · People's perception · Human interference

Introduction

Madhuca indica J. F. Gmel. (Family, Sapotaceae) is commonly known as *Mahua* tree and occurs in forest as well as in the agricultural farms. Many tribal people in Maharashtra state in India depend upon this tree species for their livelihood and food. About 80% of the juice is extracted from the flowers to produce honey-like sweets, jams, jellies, and other bakery items (Patel and Naik 2010). Moreover, the spent flowers after fermentation are used as animal feed. The estimated *M. indica* seed and flower production in the country is 0.12 and more than one million tons,

respectively (Patel et al. 2012). *M. indica* is traditionally used as a medicine for various ailments and even extensive studies show the presence of fatty acids, saponins, sugars, triterpenoids steroids, flavonoids, and glycosides (Patel et al. 2012). *M. indica* is reported to possess antioxidants, anti-inflammatory, antimicrobial, hepato protective, anti-ulcer anti-hyperglycaemic activity, antinociceptive, and anti-diarrheal activities (Chaudhary et al. 2015; Patel et al. 2012). The oil obtained from the seeds has potential as a biodiesel (Kulkarni et al. 2013) with high free fatty acids (Ghadge and Raheman 2005). Since ages, *M. indica* trees are being protected and conserved by the tribal community due to its potential for their livelihood, food security, and cultural perspective. Each protected tree is recognized for its sugar content, flower yield, time of flower dropping, proteins, and minerals in the flowers, and oil content in the seeds. Even though many trees are protected, the natural regeneration in the wild and farmland is gradually reducing, probably due to climatic conditions and human interference. The first step towards conservation of unique and important candidate *M. indica* trees would be by studying the genetic diversity

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in three regions of Maharashtra, India. Genetic diversity is important for a given species for its adaption to the changing environmental conditions, to cope up with biotic and abiotic stresses, etc. (Gienapp et al. 2008). Molecular markers are the powerful tools to detect genetic variation within and among species, varieties, and even clones (Bachmann 1994; Joshi et al. 1999).

Molecular markers such as random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSRs), amplified fragment length polymorphism (AFLP), and diversity arrays technology (DART) have been employed to access genetic diversity very frequently (Nybom et al. 2014). ISSRs have been used to study genetic diversity in spatially isolated populations (Apte et al. 2006; Bahulikar et al. 2004a), temporal populations (Bahulikar et al. 2004b), accessions of crops (Idris et al. 2012; Morikawa et al. 2014; Nagaraju et al. 2002), etc. Dinucleotide repeats have been used very frequently than tri- or tetra-nucleotide repeats to access diversity of tree species (Bahulikar et al. 2004a; Aabd et al. 2015; Naik et al. 2009; Dai et al. 2013).

So far very limited genetic diversity studies have been reported in genus *Madhuca*. Morphological markers were used to study half-sib families of candidate trees of *M. longifolia* (Syn. *M. indica*) demonstrating higher values of inter and intracluster distances (Wani et al. 2015). RAPDs were used to analyze the genetic diversity in *M. longifolia* (Gavankar and Chemburkar 2016), but the study represented only ten genotypes. ISSRs were used to assess human impact on genetic diversity of populations of *M. hainanensis* in China and demonstrated the loss of genetic structure in higher human impact locations (Dai et al. 2013). To our knowledge, there are no reports available on the genetic diversity of *M. indica* using molecular markers. This is the first study to the best of our knowledge where genetic diversity of candidate trees of *M. indica* has been studied using ISSR markers. Candidate trees which are important to local tribal community were selected on purpose from three tribal dominated regions of Maharashtra, India.

Materials and methods

Selection of the candidate trees and sampling

The candidate trees were selected specially from tribal areas of Jawhar, Dhadgaon, and Etapalli of Maharashtra, India (Supplementary Fig. 1). The three sites were at least 400 km away from each other. The work was initiated by a survey of families from 45 villages and the trees were recorded according to various parameters. Forty-eight trees showing higher flower and seed yield and nutritive quality, chosen by local tribals for their food security and livelihood, were selected as 'candidate trees'. More than 90% of the candidate trees were

located on the bunds of the farms. Fresh leaves from 48 candidate trees belong to 3 regions Etapalli (24 plants), Jawhar (10 plants), and Dhadgaon (14 plants) have been sampled (Supplementary Table 1, Supplementary Fig. 1) and stored at -70°C freezer till further use.

DNA extraction and ISSR amplification

Leaves were ground to a fine powder in liquid nitrogen. DNA was extracted from 100 mg of leaf powder using modified CTAB method (Murray and Thompson 1980). Quality and quantity of the genomic DNA were checked by 1% Agarose gel electrophoresis and diluted to 20 ng/ μl using Nanodrop (Thermo, India) for PCR amplification. Genetic fingerprinting was carried out using 14 ISSR primers (Table 1) which were selected based on distinct and reproducible banding patterns. The PCR reactions were carried out in 20 μl volume, as detailed before (Deshpande et al. 2001; Bahulikar et al. 2004a). The amplified products were separated on 2.0% agarose gel in 1.0X TAE buffer and bands were detected by Ethidium-Bromide staining. Stained gels were documented using gel documentation system (BIO-RAD, India).

Data analysis

All distinct and well-resolved fragments were scored for the presence (1) or absence (0) of bands and the binary data were used for all further analyses. Genetic parameters such as percentage of polymorphic band(s), observed number of alleles

Table 1 Numerical data as obtained from PCR amplification by 14 ISSR primers for *Madhuca indica*

Sr. no.	Primer sequence* (5'–3')	Total number of amplified loci	Total number of polymorphic loci	Percent polymorphism
1	(AG) ₈ T	9	8	88.89
2	(AC) ₈ YG	11	11	100.00
3	(AC) ₈ YT	9	9	100.00
4	(AG) ₈ YT	11	10	90.91
5	(TC) ₈ G	8	7	87.50
6	(AC) ₈ G	12	12	100.00
7	(GA) ₈ T	13	11	84.62
8	(CA) ₈ RC	12	12	100.00
9	(CT) ₈ RC	7	6	85.71
10	(CA) ₈ G	8	7	87.50
11	(CT) ₈ G	12	12	100.00
12	(AG) ₈ YC	8	6	75.00
13	(CA) ₈ A	12	11	91.67
14	(CA) ₈ G	8	7	87.50
	Total	140	129	92.14

*Y=C or T pyrimidine and R=A or G purine

(N_a), the effective number of alleles (N_e), Nei's genetic diversity (H), Shannon's information index (I), total genetic diversity (H_t), genetic diversity within regions (H_s), Nei's genetic differentiation index among regions (G_{st}), and gene flow estimates between regions (N_m) were determined with POPGENE version 1.32 (Yeh et al. 1999). Variance within the region was analyzed as Analysis of Molecular Variance (AMOVA) and Nei's heterozygosity of the regions using GenALEX version 6 (Peakall and Smouse 2006, 2012). Genetic distance matrix obtained from GenALEX was used to construct a dendrogram by neighbor-joining method by importing it into MEGA ver.6.06 (Tamura et al. 2013). The principal coordinate analysis (PCA) was analyzed using PAST 3 (Hammer et al. 2001).

Results and discussions

In this study, ISSR primers were used which proved to be useful due to their low operating cost, high polymorphism, high reproducibility and have been used to study variation in phylogenies (Mukherjee et al. 2010).

In the present study, 14 different dinucleotide repeat primers yielded a total of 140 bands and overall 92% polymorphism (Table 1) corresponding to an average of nine polymorphic bands per primer. The highest number of bands was recorded by the primer (GA)₈T (13) and lowest by (CT)₈RC (7). Highest polymorphism (100%) was observed by three primers (AC)₈YG, (AC)₈YT and (CT)₈G, whereas the lowest by (AG)₈YC (75%). The sequence repeats of the primers (AG)_n, (AC)_n, (CA)_n, (GA)_n, and (TC)_n used in the present study have been useful for analysis of genetic diversity in several tree species (Aabd et al. 2015; Apte et al. 2006; Bahulikar et al. 2004a).

A high level of polymorphism has been reported in another species from the same genus *Madhuca*, *M. hainanensis* (96.9%) (Dai et al. 2013) and in other genera such as *Eurya* (Bahulikar et al. 2004a) and *Terminalia* (Sarwat et al. 2011). Region-wise percent polymorphism was highest for Etapalli (83.57%) and lowest for Dhadgaon (45.00%) with an average of 65.71% (Table 2). In this study, percent polymorphism obtained for Etapalli was more than the values obtained for protected regions and similar observations have been made for *M. hainanensis* (Dai et al. 2013), whereas the values of polymorphism recorded for Jawhar and Dhadgaon are in accordance as reported for higher human impact region (Dai et al. 2013). As the trees in Jawhar and Dhadgaon are located on field bunds, human interference would have been higher at these places compared to Etapalli which has higher forest cover.

Genetic diversity for *M. indica* trees was recorded in terms of various indices. Number of alleles (N_e), observed number of alleles (N_a), Nei's genetic diversity (H), and Shannon's information index (I) (Table 2) was 1.921, 1.333, 0.211, and 0.337, respectively. These values were lower

Table 2 Genetic diversity within regions and genetic differentiation parameters of *M. indica*

Region	N_a	N_e	h	i	PIC
Etapalli	1.764	1.327	0.2057	0.3261	83.57
Jawhar	1.479	1.321	0.1962	0.3052	68.57
Dhadgaon	1.093	1.234	0.1397	0.2128	45.00
Average	1.445	1.294	0.1805	0.2814	65.71
Species level	1.921	1.333	0.211	0.337	92.14

N_a observed number of alleles, N_e effective number of alleles, h Nei's gene diversity, i Shannon's information indices, PIC polymorphic information content

compared to *M. hainanensis* (Dai et al. 2013). The total genetic diversity (H_t) and genetic diversity within a region (H_s) were 0.2081 and 0.1805, respectively, which was very low compared to *M. hainanensis* (Dai et al. 2013).

Region-wise analysis demonstrated higher values for Etapalli and lowest for Jawhar (Table 2). Region-wise analyses revealed overall lower polymorphism, genetic variation, and heterozygosity. It also consistently showed lower values for Dhadgaon and higher values for Etapalli suggesting that trees from Etapalli were more diverse than the other two regions.

Similarly, region-wise lower variation was observed in *M. hainanensis* (Dai et al. 2013).

AMOVA revealed 15% genetic variation among regions which was in consideration with G_{st} , a measure of genetic differentiation among population (Holsinger and Weir 2009). Genetic variance was highest (85%) within individuals (Table 3). The results are higher than *M. hainanensis* where among-population and within-population variance was 12 and 71%, respectively (Dai et al. 2013). *M. indica* is a long-living tree having features specific for trees such as overlapping generations and a long juvenile phase (Austerlitz et al. 2000). Additionally, it is a cross-pollinated species having strong self-incompatibility (Heslop-Harrison 1975). Therefore, values reported in this study were in agreement with the values which have been reported for cross-pollinated and long living species (Nybom 2004). As relatively high gene flow ($N_m = 3.28$) was detected, possibility of higher genetic variation within populations due to long-range gene movements might hold true (Nybom and Bartish 2000).

The neighbor-joining dendrogram was constructed based on Nei's genetic distance resulted in three main clusters. Cluster 1 was the innermost cluster represented by trees from all three regions. It was also showing a grouping of 12 plants from Dhadgaon. Cluster 2 exhibited two sub-groups, where cluster 2a represented seven trees from Etapalli and 2b represented two trees from Etapalli and four trees from Jawhar. Cluster 3 composed of trees only from Etapalli. Overall dendrogram indicated the presence of no geographical structuring (Fig. 1). Principal Coordinate Analysis (PCA) confirms the absence of geographical structuring and

Table 3 Analysis of molecular variance (AMOVA) of *Madhuca indica*

	Source	df	SS	MS	Est. Var.	%	P (rand $\geq$ data)
Overall	Among regions	2	109.831	54.915	2.070	15	0.001
	Within individuals	45	693.336	15.407	15.407	85	0.001
	Total	47	803.167		18.044	100	

Fig. 1 A neighbor-joining cluster analysis for 48 candidate trees of *Madhuca indica*; regions are color coded

Fig. 2 Principal co-ordinate analysis of the 48 candidate trees of *Maduca indica* based on 14 ISSR primers

shows intermixing of the convex hulls of each region from the study area (Fig. 2).

The coefficient of genetic differentiation among populations (G_{ST}) was 0.1324 suggesting 13.24% variation. *Madhuca* is a member of the family Sapotaceae and we observed that this value was lower than that of another Sapotaceae member *Argania spinosa* (Aabd et al. 2015) and it was higher than *Gmelina arborea* studied from India (Naik et al. 2009). G_{ST} indicated a low level of genetic differentiation among populations. In *Eurya nidita* very high G_{ST} was reported (0.49) where populations showed strict spatial clustering (Bahulikar et al. 2004a).

Overall analysis demonstrated no geographical structuring in *M. indica*. Similarly, no geographical structuring has been observed in *M. hainanensis* (Dai et al. 2013) and in *M. longifolia*, although the sample size was too small (Gavankar and Chemburkar 2016). The reasons behind the absence of geographical structure mentioned for *Salix* sp. were presence of intermediate populations or high-speed dispersal or high mutation rates (Palme et al. 2003) which might also hold true for candidate trees of *M. indica* in the present study.

Higher diversity, small genetic variation among-population, and no correlation between geographical distance and genetic variation suggest a human impact on habitat (Chen et al. 2014). The present study also suggests similar trends as the plants were sampled based on the need of the tribal community. Further research is needed where genetic diversity of Mahua sampled from undisturbed forests could be compared to the genetic diversity data obtained from present study, to judge how diversity can be affected by human activities.

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Author contributions SDN, VKK, and SA gave technical guidance and were involved in documentation, identification, and collection of plant samples. SSJ and RAB executed the laboratory experiments and RAB interpreted and wrote the draft manuscript. All the authors scrutinized and reviewed the manuscript, and approved the final version.

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Compliance with ethical standards

In present work, human participation was limited to identification of plants by local tribal people and, therefore, formal consent was not required.

Conflict of interest The authors declare no conflict of interests.

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