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Determination of Genetic Diversity of Tamarind (*Tamarindus indica*) Accessions in Eastern Region of Kenya Using Inter Simple Sequence Repeat (ISSR) Markers

Mercy Liharaka Kidaha [a++*], Cornelius Wainana Mbathi [a++] and Fredah Rimberia Wanzala [a++]

[a] Department of Horticulture and Food Security, Jomo Kenyatta University of Agriculture and Technology, Kenya.

Authors' contributions

This work was carried out in collaboration among all authors. All authors contributed to the study design and concept. Author MLK did the fieldwork, lab work, data analysis, interpretation and manuscript preparation. Author CWM did the data analysis and corrections of the paper. Author FRW did the field study and paper correction. All authors read and approved the final manuscript.

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ABSTRACT

There is scanty information on tamarind's genetic diversity in Easter Kenya. The objective of this study was to determine the genetic diversity of 64 tamarind accessions from Eastern Kenya using 12 Inter Simple Sequence Repeat (ISSR) markers. DNA was extracted from the young apical leaves using modified CTAB method and amplified using standard PCR. The data collected were

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[++] *Ph.D;*
**Corresponding author: E-mail: mlihaaraka@gmail.com;*

scored as presence (1) or absence (0) of bands then compared to the 100bp ladder and analyzed using GeneAlex and R softwares. Only seven primers produced reproducible bands. A total of 46 alleles were produced for the 7 loci with an average of 6.6 per loci. Polymorphic information content (PIC) varied from 0.72 to 0.89 and genetic diversity varied from 0.74 to 0.9. The ISSR markers revealed effective polymorphism of 40.87 to 99.46% and the band sizes varied from 100 to 1000 bp. Analysis of Molecular Variance (AMOVA) depicted high variation within the tamarind populations at 90% and the least variation of 10% among the population. The first 3 components of Principal coordinate analysis (PCoA) contributed 40.83% of the total variation. Hierarchical cluster analysis grouped the tamarind accessions into seven major distinct groups. Tamarind accessions were different within counties with minimal variations among counties, proving that genetic diversity exists among the tamarind accessions in the Eastern region of Kenya. High genetic diversity was evident among Embu accessions and least among Masinga accessions. Diversity in tamarind can be utilized in marker-assisted breeding and primer ISSR17899A and ISSRHB11 can be explored in studying genes that code for various traits in tamarind accessions.

Keywords: Genetic diversity; Kenya; ISSR; Tamarindus indica.

1. INTRODUCTION

Tamarind (*Tamarindus indica*) is a leguminous, evergreen perennial tree that is native to Africa and Asia and it is adaptable to the tropic and subtropics; Additionally, it is highly tolerant to drought [1]. Tamarind pulp is utilized in food, pharmaceuticals, textile, cosmetic, oil, paper, and printing industries [2,3]. Tamarind leaves are sources of vegetables, during the lean periods and have been reported to contain vitamins and minerals such as calcium, iron, and ascorbic acid [4]. The tamarind tree is grown in home gardens, farmlands, roadsides, and on common lands [5]. The tree is commonly grown from seeds of unknown parentage and this has resulted in wide variation among the progenies. The wide genetic variation is also aided by the large geographical distribution, adaptation, and cross-pollination [5;6]. Trees with wide variation within the population are preferred in selecting the best trees in relation to crop improvement [6]. Very little is known about tamarind's genetic improvement, and farmers choose cultivars based on observable desirable traits, especially the taste and color of the pulp [5]. These traits are highly altered by environmental factors and have many limitations in perennial crops [5]. Very little has been studied on tamarind conservation, genetic characteristics, and population biology [7].

Characterization based on DNA molecular markers is more reliable and not affected by environmental factors [8]. A clear and detailed study of the molecular diversity of Kenyan tamarind has not been carried out. Molecular characterization has been carried out in Bangalore, India, Burkina Faso, and Ecuador using AFLPs, RAPDs, and ISSRs, respectively [5;9;10;7]. ISSRs are highly polymorphic, simple, and reproducible and use a primer length of 16-25 mers [11]. ISSR markers have been used in characterization studies and have revealed genetic diversity in various crops: *Opuntia* [12], Hassawi rice [13], in cucumber [10], and tamarind [7]. The results of this study will enhance tamarind improvement through conventional breeding, utilization and spearhead gene characterization.

2. MATERIALS AND METHODS

2.1 Sampling

Field survey was carried out from December 2015 to August 2016. A total of 64 tamarind accessions were collected from 4 counties in Eastern region of Kenya. Ten samples were collected from Mwingi, 21 from Ishiara (Embu), 6 from Masinga, and 27 from Kibwezi. These locations are in Kitui, Embu, Machakos and Makueni counties, respectively. Accessions from Mwingi were labelled as MW001- MW010, Embu as E001-E021, Masinga as MS001-MS006 and Kibwezi as KB001-KB027.

2.2 Sample Preparation and DNA Extraction

Apical young leaves were collected and placed in falcon tubes containing silica gel and transported to molecular biology laboratory at the institute for Biotechnology of Jomo Kenyatta University Kenya. The leaves were crushed in liquid nitrogen and stored for further extraction as described by [14]. DNA extraction was done

using 0.4 g of leaves that were ground in 3ml of extraction buffer (CTAB) as described by [14]. The CTAB buffer contained (1M Tris HCL (pH 8), 0.5M (EDTA) (pH 8.0), 5M (NaCl), (Na_2SO_4), (PVP10) and 2% CTAB and then incubated at 65°C for 30 min. The samples were then centrifuged at 13,000 revolutions per min (rpm) for 12 min and the supernatant mixed with equal volumes of chloroform: Isoamyl alcohol (24:1). The mixture was then centrifuged at 13,000 rpm for 10 min and the chloroform: Isoamyl step was repeated. The supernatant was mixed with equal volumes of cold Isopropanol and incubated at room temperature. The nucleic acid was pelleted at 13,000 rpm for 5 min and then washed with 70% ethanol twice. The pellet was air-dried and re-suspended in 50 µl of deionized water. Visualization gel was prepared using 0.8g of agarose in 100ml of (TBE) buffer and heated for 2 min using microwave and ethidium bromide (EtBr) added. Loading dye of 3 µl was mixed with 7 µl of re-suspended pellet in deionized water and loaded. Observations were made using a bench top UV trans illuminator and the presence as (1) and absence as (0) of bands was scored after 45 min.

2.3 PCR Reaction

DNA was amplified using ISSR primers described by [7] in (Table 1). Each 20 µl of PCR mix comprised of 10 µl of 2X Bioneer ready mix with 2 µl of primer, 2 µl of DNA and 6 µl of PCR water and a negative control without the DNA template. Twelve primers were used to screen for polymorphic primers. The PCR reaction was as follows initial denaturation at 94˚C for 5 min, denaturation at 94˚C for 30 sec annealing at 54 to 44˚C (touch down PCR) for 1 min, extension at 72˚C for 2 min and final extension at 72°C for 7 min as described [7]. Amplified DNA was visualized on 2% agarose on gel documentation system and the band sizes estimated by comparing with a 100 bp DNA ladder.

2.4 Data Analysis

Data from ISSR primers were generated by scoring (1) for presence and (0) for absence of bands. The binary data was used to obtain polymorphic information content (PIC) according to Liu *et al.,*2011. PIC=1- $\sum_{j-1}^{n} P2ij$ where $\mathbf{P_{ij}}$ is the frequency of the $\mathbf{j^{th}}$ allele for i^{th} locus and summation extends **n** alleles scored for ISSR locus. Genetic diversity was obtained using genotypic richness (number of multilocus genotypes observed per population, MLG). Genotypic diversity was estimated as the percentage of polymorphism observed by each population %Pj, Shannon Weiner index of MLG diversity per population. Simposons index per population Lambda, Evenness index per population –E. Expected heterozygosity or unbiased gene diversity for each population –He. Observed heterozygosity per population –Ho were analyzed using R3.6.3 software.

Genalex 6.5 software (Peakall & Smouse, 2012) was used to determine Principal Coordinate Analysis (PCoA), Analysis of Molecular Variance (AMOVA) to give the difference between populations and between the accessions. The data were then subjected to R software to obtain phylogenetic clusters using Hierarchical cluster analysis. Accessions from Mwingi were denoted as population 1, Masinga denoted as population 2, Kibwezi as population 3 and Embu as population 4.

3. RESULTS

3.1 Selection of Polymorphic Primers from Candidate ISSR Primers

Touch-down PCR was used for optimization with annealing temperatures of 54 to 44 ˚C for 35 cycles. Primers ISSR 807, ISSR 836, ISSR 842, ISSR 844, ISSR HB11, ISSR 17899A and ISSR 17899B produced reproducible bands (Table 2), while primers ISSR 808, ISSR 814, ISSR 835, ISSR848 and ISSR 860 did not amplify DNA products.

The seven scorable primers resulted in 7 loci with a total of 46 alleles. The average number of alleles was 6.6 alleles per locus. The alleles ranged from 5 for 807 to 10 alleles for ISSRHB11. ISSRHB11 had the highest polymorphism of 0.89 and the highest gene diversity of 0.90. ISSR807 showed the least polymorphism of 0.73 with the least gene diversity of 0.74 (Table 3).

3.2 Analysis of Molecular Variance (AMOVA)

Analysis of molecular variance depicted higher variation within a population than among populations. Variation within a population was 90%, while among the population was 10% (Table 4). Principal coordinate analysis showed that the first three components of two-dimensional PCoA contributed 40.83% of the

total variation (Table 5). Accessions in populations 1, 2, 3 (Mwingi, Masinga and Kibwezi) were closely related, while accessions from Embu were further apart (Fig. 1).

3.3 Cluster Analysis of Tamarind Accessions from Semi-Arid Eastern Kenya

HAC clustered the 64 accessions into 7 major clusters. Cluster one comprised of accessions from Embu which included; E008, E001 and E009. Cluster two comprised of accessions from Mwingi only and one from Masinga which include; MW009, MW008, MW010, MW006, MW007, MW005, MW002, MW003 and MS004. Cluster 3 comprised of accessions from Embu and Masinga which included; E011, E012, E013, E021, E003, MS003 and E014. Cluster four comprised of accessions from Kibwezi which included; KB004, KB005, KB006, KB012, KB015, KB001, KB010, KB011, KB002 and KB007. Cluster five comprised only one accession from E010. Cluster six comprised of accessions from Kibwezi and Masinga which included; KB020, MS01, KB008, KB017, MS002, KB009, MS006, KB024, KB021, KB024, KB027, KB023, KB019, KB025, KB002, KB013 and KB014. The last cluster seven comprised of accessions from Embu and Kibwezi which included E015, E016, KB022, KB018, KB026, E005, E017, KB019, KB020, KB004, KB006, KB003 and E018.

Table 1. ISSR primers used in characterization of 64 tamarind accessions collected from Eastern region of Kenya

S/NO	Name	Sequence	Type of nucleotide
1	ISSR807	AGA GAG AGA GAG AGA CT	Tri nucleotide
2	ISSR814	CTC TCT CTC TCT CTC TA	Tri nucleotide
3	ISSR836	AGA GAG AGA GAG AGA GCTA	Tri nucleotide
4	ISSR860	TGT GTG TGT GTG TGT GAGA	Tri nucleotide
5	ISSRHB11	GT GT GT GT GT GT CC	Di-nucleotide
6	ISSR808	AGA GAG AGA GAG AGA GC	Tri nucleotide
7	ISSR844	CT CT CT CT CT CT CT CT AC	Di- nucleotide
8	ISSR835	AGA GAG AGA GAG AGA GCTC	Tri nucleotide
9	ISSR17899A	CA CA CA CA CA CA AG	Di-nucleotide
10	ISSR17899B	CA CA CA CA CA CA GG	Di- nucleotide
11	ISSR848	CAC ACA CAC ACA CAC AGC	Tri nucleotide
12	ISSR 842	GAG AGA GAG AGA GAG ACTG	Tri nucleotide

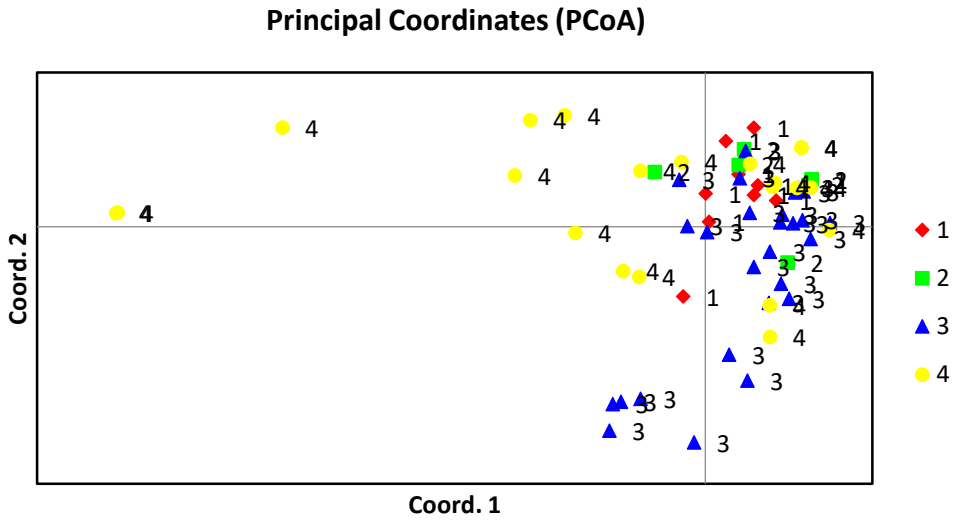

Fig. 1. Principal coordinate analysis (PCoA) of tamarind populations from semi-arid Eastern Kenya (1-Mwingi, 2-Masinga, 3-Kibwezi, 4- Embu)

Table 1. Analysis of polymorphism obtained using 7 ISSR primers in 64 tamarind accessions from Eastern region of Kenya

ISSR Primers	Number of amplified loci(a)	Number of polymorphic loci (b)	Effective Polymorphism %	Min band	Max band
ISSR807	43	32	40.87	200	800
ISSR836	72	61	78.33	400	800
ISSR842	78	66	84.76	300	700
ISSR844	83	57	73.24	200	900
ISSR17899A	80	63	80.92	100	800
ISSR17899B	103	79	99.46	100	1000
ISSRHB11	86	68	87.33	100	1000

Table 2. Analysis of loci and the total number of allele frequencies using the ISSR primers from Eastern region of Kenya

Loci	Allele	1-D (PIC)	Hexp	Evenness
ISSR807	5	0.73	0.75	0.84
ISSR836	5	0.74	0.75	0.87
ISSR844	6	0.77	0.78	0.83
ISSR842	5	0.78	0.79	0.94
ISSR17899A	6	0.83	0.83	0.97
ISSR17899B	9	0.89	0.89	0.98
ISSRHB11	10	0.89	0.90	0.96
Mean	6.6	0.80	0.81	0.91
Total	46			

Key. 1-D = Simpson index (Simpson, 1949), Hexp = Nei's 1978 gene diversity

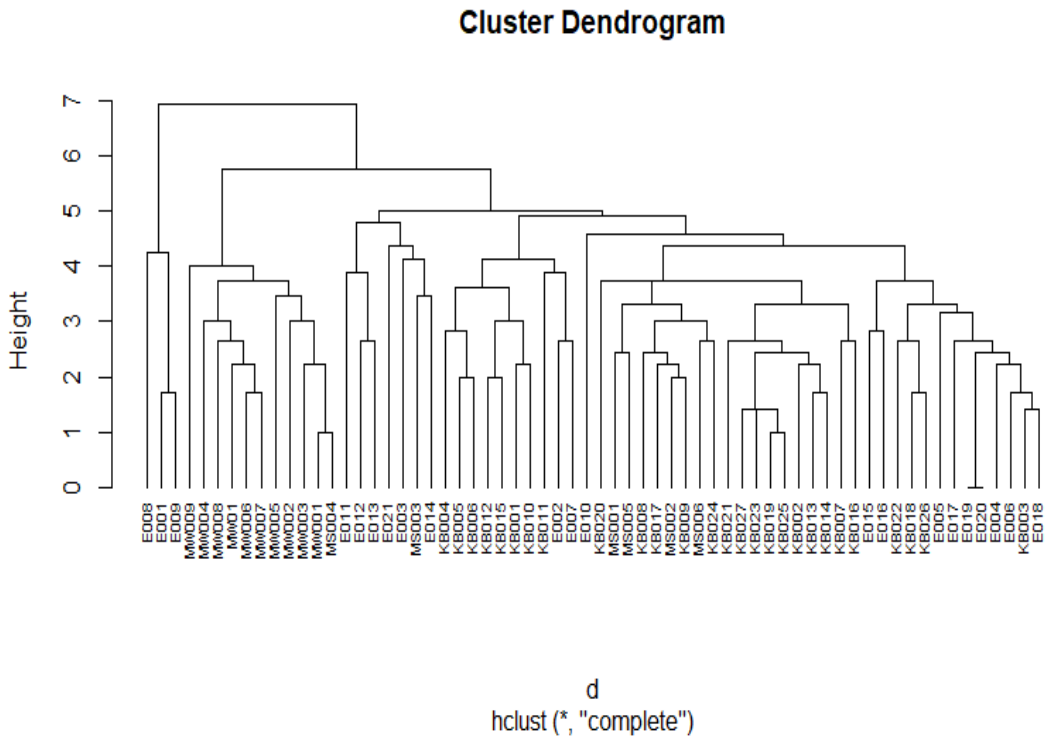

Fig. 1. HAC dendrogram of 64 tamarind accessions from Eastern region of Kenya amplified using 7 ISSR markers

Table 4. Analysis of molecular variance in 64 tamarind accessions

Source	df	SS	MS	Est. Var.	%
Among Pops	3	49.76	16.59	0.71	10%
Within Pops	60	374.85	6.247	6.25	90%
Total	63	424.6		6.96	100%

Table 5. Principal coordinate analysis of 64 tamarind accessions

Axis	1	2	3
%	21.20	11.05	8.58
Cum %	21.20	32.25	40.83

4. DISCUSSION

Self-incompatible plants have genetic differences at species level and lower differences among populations [15]. Genetic differences in tamarind were expected since the plant is cross-pollinated and propagated using seeds thereby displaying significant variation within the populations [16]. The presence of pollinators promotes diversity and decreases inbreeding. Sufficient pollinators promote gene flow which in turn promotes diversity [17]. Plants with high geographical ranges tend to maintain high genetic diversity than geographically localized species [18]. Genetic diversity within a population is also influenced by population size, genetic drifts, gene flow and extended periods with a low number of individuals [19;20]. When the population size is large the genetic variation is also high and the plants can adapt to climatic changes unlike small populations which are threatened by genetic drifts that led to inbreeding depression and loss of diversity [21]. Extended long periods with a low number of individuals in an area can also minimize diversity. Most of the accessions clustered across the counties which was supported by the fact that the tamarind tree is self-incompatible [22] and propagated using seeds [23]. The presence of pollinators that promote gene flow within populations, tamarind populations are still large. This clustering was contrary to reports by [24] who reported that plum varieties evaluated clustered based on the regions of study. Tamarind is a perennial tree and also is able to maintain high levels of variation compared to annuals and short-lived perennials [25]. High levels of variation were also associated with the fact that the tree was able to adapt to different environmental conditions [5]. Tamarind populations were genetically isolated by mutation and genetic drift that lead to differences in the allele frequencies at selectively natural loci. The least diversity was observed in Masinga and this is attributed to habitat loss, small population, degradation, exploitation and introduction of crop plants in the region.

5. CONCLUSION

Genetic diversity was revealed among the tamarind accessions in Eastern region of Kenya. Populations from Embu showed greater diversity as they clustered in 10 groups and PCoA they clustered differently and far away from the rest, while the least diversity was observed in Masinga and Mwingi population.

6. RECOMMENDATION

High diversity in Embu can be exploited in marker-assisted breeding. High PIC produced by primer ISSR17899A and ISSRHB11 can be used to study the genes that code for important traits in tamarind.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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