Original Research Article

EVALUATION OF GENETIC DIVERSITY OF TAMARIND (*Tamarindusindica*) ACCESSIONS IN EASTERN REGION OF KENYA USING ISSR MARKERS

Comment [A1]: Intersimple sequence repeat (ISSR)

Abstract

There is limited information on the genetic diversity of tamarind in Kenya. The objective of this study was to evaluate the genetic diversity of 64 tamarind accessions from Eastern Kenya using 12 Inter Simple Sequence Repeat (ISSR) markers. DNA was extracted from apical leaves using the CTAB method and amplified using standard PCR. ~~Data~~ The collected data were scored as presence (1) or absence (0) of bands and analyzed using GeneAlex and R softwares. Seven ISSR primers produced reproducible bands. A total of 46 alleles were produced for the 7 loci with an average of 6.5 per loci. Polymorphic information content (PIC) varied from 0.72 to 0.89 and genetic diversity of 0.74 to 0.9. The ISSR markers revealed effective polymorphism of 40.87 to 101.46% and the band sizes varied from 100-1000 bp. Analysis of Molecular Variance (AMOVA) revealed high variation within the tamarind population at 90% and the least variation among the population at 10%. Principal coordinate analysis (PCoA) revealed that the first three components contributed 40.83% of the total variation. Cluster analysis showed that tamarind accessions were diverse and were grouped into seven major distinct groups. Tamarinds were different within counties, but the variations were minimal among counties, proving genetic diversity exists among the tamarind accessions in the Eastern region ofKenya.

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Keywords: ~~AMOVA~~, Genetic diversity,Kenya, ISSR, ~~PCoA,~~ *Tamarindusindica*

Introduction

Tamarind (is a dicotyledonous, 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 used in food, pharmaceuticals, textile, cosmetic, oil, paper, and printing industries [2,3]. Tamarind leaves are used as vegetables and are reported to contain vitamins and minerals such as calcium, iron, and ascorbic acid [4]). Tamarind is grown in home gardens, farmlands, roadsides, and on common lands [5]. This tree is commonly grown from seeds of unknown parentage and this has resulted in wide variation among the progenies. Wide genetic variation is also aided by the large geographical distribution, adaptation, and cross-pollination nature of the tree [5;6]. Trees with wide variation within the population offer opportunities for selecting the best trees in relation to crop improvement [6]. Very little is known about the genetic improvement of tamarind and farmers choose cultivars based on observable desirable traits especially the taste and color of the pulp [5]. These observable 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]).

Comment [A4]: Add the scientific name

Characterization based on DNA 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 done. Molecular characterization has been carried out in Bangalore, India, Burkina Faso, and Ecuador using AFLPs, RAPDs, and ISSRs, respectively [5;9;13;7]

ISSRs are highly polymorphic, simple, and reproducible and use a primer length of 16-25 mers [10]. ISSR markers have been used in characterization studies and revealed genetic diversity in *Opuntia*[11]), Hassawi rice [12], in cucumber [13], and tamarind [7].

What is the significance of this study

Materials and methods

Sampling

Field survey was carried out from December 2015 to August 2016. A total of 64 tamarind~~samples~~ were collected, 10 samples from Mwingi, 21 from Ishiara, 6 from Masinga, and 27 from Kibwezi. These locations are in Kitui, Embu, Machakos and Makueni counties, respectively.

Passport data of the 64 accessions ~~shoud~~should be listed here in table

Sample preparation and DNA extraction

Apical leaves were collected and placed in falcon tubes containing silica gel and transported to the laboratory. 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 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 was mixed with equal volumes of chloroform: Isoamyl alcohol (24:1). The mixture was centrifuged at 13,000 rpm for 10 minand 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 minand then washed with 70% ethanol twice. The pellet was air-dried and re-suspended in 50 µl of sterile distilled water. Visualization gel was prepared by weighing 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 distilled water and loaded. Observations were made and the presence and absence of bands was scored after 45 min.

PCR reaction

DNA amplification was done using ISSR primers as 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. Twelve primers were used to screen for the more 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 for 1 min, extension at 72˚C for 2 min and final extension at 72˚C for 7 minas described [7].Amplified DNA was visualized on 2% agarose. Band sizes were estimated by comparing with a 100 bp DNA ladder.

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

S/NO	Name	Sequence include the annealing temperature of each primer and indicate are they di,tri,tetranuclecotide
1	ISSR807	AGA GAG AGA GAG AGA CT
2	ISSR814	CTC TCT CTC TCT CTC TA
3	ISSR836	AGA GAG AGA GAG AGA GCTA
4	ISSR860	TGT GTG TGT GTG TGT GAGA
5	ISSRHB11	GT GTGTGTGTGT CC
6	ISSR808	AGA GAG AGA GAG AGA GC
7	ISSR844	CT CTCTCTCTCTCTCT AC
8	ISSR835	AGA GAG AGA GAG AGA GCTC
9	ISSR17899A	CA CA CA CA CA CA AG
10	ISSR17899B	CA CA CA CA CA CA GG
11	ISSR848	CAC ACA CAC ACA CAC AGC

Comment [A5]: Better to say accessions

Comment [A6]: Which laboratory?

Comment [A7]: Specify the gel documentation type used to obserb the bands

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12	ISSR 842	GAG AGA GAG AGA GAG ACTG

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.

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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 ~~obtained~~ data were 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.

Results

Selection of polymorphic primers from candidate ISSR primers

Optimization was done using touch-down PCR at 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.

Table 1~~2~~: 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	101.46	100	1000
ISSRHB11	86	68	87.33	100	1000

The seven scorable primers resulted in 7 loci with a total of 46 alleles. The average number of alleles was 6.~~5~~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).

Table 2~~3~~: Analysis of loci and the total number of allele frequencies using 7 ISSR primersfrom 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: ~~allele = Number of observed alleles,~~ 1-D = Simpson index (Simpson, 1949), Hexp = Nei's 1978 gene diversity, ~~Evenness of allele distribution~~

Analysis of molecular variance (AMOVA).

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

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

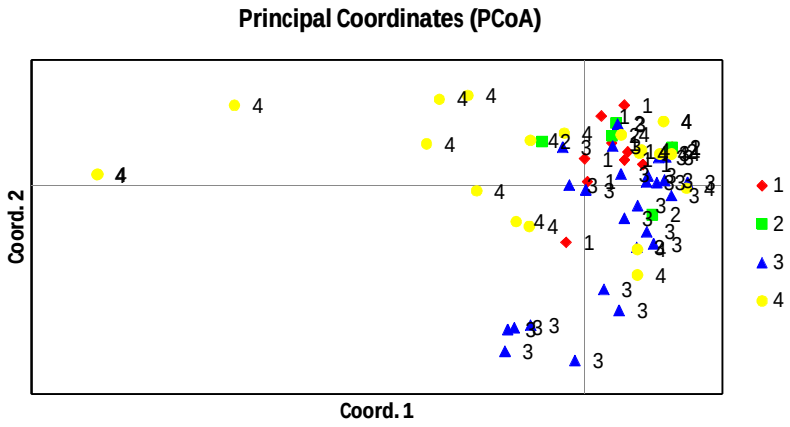

Figure 1: Principal coordinate analysis (PCoA) of tamarind populations from semi-arid Eastern Kenya (1-Mwingi, 2-Masinga, 3-Kibwezi, 4- Embu)

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.

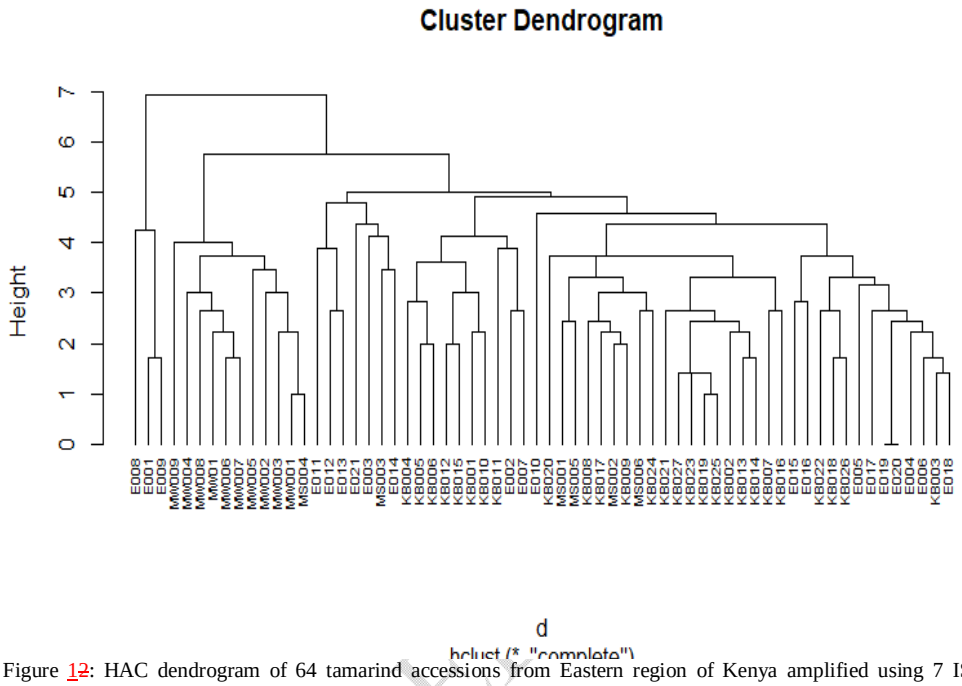

Figure 12: HAC dendrogram of 64 tamarind accessions from Eastern region of Kenya amplified using 7 ISSR markers.

Discussion

Self-incompatible plants have genetic differences at species level and lower differences among populations [15]. Genetic differences in tamarind were expected since it is a cross-pollinated plant and propagated using seeds thereby displaying significant variation within the population [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 Masinga and this is attributed to habitat loss, small population, degradation, exploitation and introduction of crop plants in the region.

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 least diversity was observed in Masinga and Mwingi population.

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 encode for important traits in tamarind.

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Comment [A11]: Include the numbers

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