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Molecular characterization of diversity in *Annona squamosa* L.: A high value tropical medicinal plant

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Abstract

Introduction: *Annona squamosa* L. commonly known as custard apple, is an important under-utilized fruit species from tropical and subtropical parts of India with high potential as commercial horticultural crop. The aim of this study was to determine the genetic diversity among populations spread across districts of Udaipur, Rajsamand and Chittaurgarh falling within Aravallis in Rajasthan.
In all, 21 populations were identified and subjected to molecular evaluation using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. The cumulative analysis carried out on the basis of RAPD and ISSR data sets revealed 73.91% polymorphism.

Methods: A total of 251 amplicons were produced using 19 RAPD and 15 ISSR primers. The pair-wise distance matrix calculated by Dice's co-efficient showed a distance range of 0.67 to 0.95 when computed using cumulative data set.

Results: Characterization of genetic diversity within populations based on combined data set showed that percentage of polymorphism bands (PPB) ranged from 29.19 to 50.93. Nei's gene diversity (H_E) values varied from 0.1002 to 0.1964 within a total genetic diversity (HT) of 0.2509. The average observed number of alleles per locus (Na) was 1.3602 and Ne was 1.2400. The Shanon's index was 0.2002 (Hpop) and 0.3800 (Hsp) at population and species level, respectively.

Conclusion: The moderate gene flow value (Nm=0.59) and significant coefficient of genetic differentiation (G_{ST}=0.458) revealed low level of genetic diversity within population and highly significant genetic differentiation among populations of *A. squamosa*. The UPGMA dendrogram resolved all the 21 populations representing 3 districts into various clusters according to their genetic distances. The principal component analysis (PCA) supported the UPGMA results and justified the relationship among populations.

Keywords: *Annona squamosa*, RAPD, ISSR, Genetic diversity

Introduction

Annona squamosa L. commonly known as "custard apple" or "sugar apple" belongs to family Annonaceae. It is also known for its delicious sweet fruits which contain creamy or custard like granular pulp rich in sugar contents. The fruit of *A. squamosa* is an important diet for tribals and most indigenous populations in India (Figures 1and 2). It is one of the most important wild fruit plant having great medicinal value. Dried powdered leaves are regarded as purgative and as a remedy for mucous diarrhoea. Venereal diseases and intestinal disorders are treated with preparations of the roots.[1,2] Leaves are rich in aporphines and fruit contain diterpenoids. Leaves, roots, bark, fruit and seeds of annonas contain numerous bioactive chemical substances, such as acetogenins, alkaloids, terpenes, flavonoids and oils. The most important acetogenins are

anonins or anonacins. This substance have toxic effects when eaten by insects and can inhibit insect growth, development and reproduction. The fruits are an excellent source of vitamin C, a source of dietryfibre as well as useful source of minerals such as Mg and K.[2]

Custard apple has been reported to have originated in tropical America from where it has gone to other parts of the world including Asia. In India it is distributed throughout tropical and subtropical parts including Rajasthan where it is not cultivated but grows wild in the districts falling in Aravalli hill region. The districts with heavy to very heavy populations of *A. squamosa* include Udaipur, Chittaurgarh and Rajsamand (Figure 3). There is a great degree of variability among the natural populations of *A. squamosa* with respect to quality and yield of fruits and other parameters. This variability among populations

Figure 1. *Annona squamosa* L. Tree Growing in Natural Habitat.

Figure 2. Fruit of *Annona squamosa*.

Figure 3. Study Areas Representing Different Districts Falling Under Aravalli Hill Region & Different Agro-Climatic Conditions of Rajasthan.

is essential for the long term survival of tree species. For molecular analysis of genetic diversity in *Annona cherimola* Mill. 16 SSR (simple sequence repeat) loci were used to find polymorphism among 279 accesion.[3] Studies on genetic structure and diversity of *A. squamosa* has been done by Salazar et al.[4] Molecular characterization of diversity of 4 natural *A. crassiflora* Mart. populations through random amplified polymorphic DNA (RAPD) markers has been done by Cota et al.[5]

Being an out breeder species predominantly, *A. squamosa* may be expected to maintain high level of genetic diversity in nature. Characterization of genetic diversity is a prerequisite for efficient conservation and utilization of genetic resources. Characterization of genetic diversity in *A. squamosa* on the basis of morphological characters have been identified.[6] It is well known that morphological characters are known to be influenced by environmental conditions. However, these observations alone cannot determine genetic differentiation of populations since differences may result from phenotypic or genetic variations.[7] An evaluation with a reliable method like molecular markers would be useful for these observations. Among several markers available RAPD[8] and inter simple sequence repeat (ISSR) markers are useful for diversity analysis.

RAPD and ISSR markers have been widely used in studies related to genetic diversity analysis of several plant species. RAPD has been used extensively for classification of accession, identification of cultivars and diversity estimation. RAPD markers provide a quick and efficient screening of DNA as they require small amount of DNA, involve non-radioactive assay, need a simple experimental set-up, do not require species-specific probe libraries and
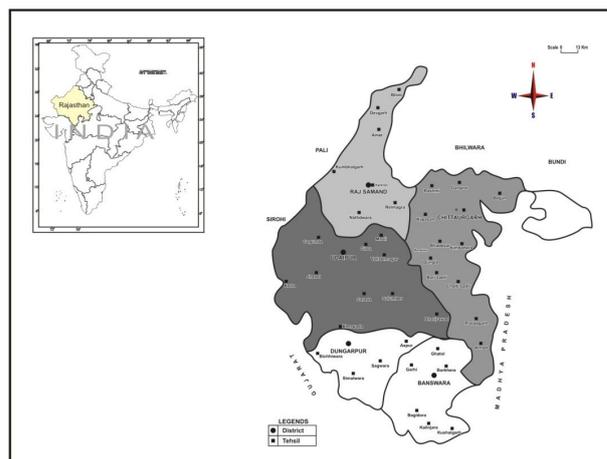
blotting or hybridization step.[9] RAPD technique has been employed to asses genetic diversity within and among natural populations of a large number of fruit tree species like Mango[10,11] Pistachio[12] Cashew[13-17] and Apricot.[18] ISSR markers are PCR based like RAPD but are more reproducible than RAPD due to their better stringency (high annealing temperature), require no gene sequence information and targets microsatellite motif. ISSR markers have been widely used for diversity analysis.[19-23]

In the present investigation RAPD and ISSR markers have been used to assess genetic variability among natural populations of *A. squamosa* occurring in three districts falling in Aravallis of south-east Rajasthan. The objective was to generate useful data which could be useful in management, improvement and conservation of *A. squamosa* germplasm.

Materials and Methods

Survey and Collection of Plant Material

Field surveys were undertaken in all the three districts for identification of localities where populations of *A. squamosa* are dominant (Figure 3). For the present study 21 localities representing twenty one populations from three districts of Rajasthan falling in Aravalli hill region were chosen for collection of *A. squamosa* samples (Table 1). Three plants were selected in each area. The populations were separated by the ranges of Aravallis which obstructed their continuity and were identified using local knowledge and previous reconnaissance. Only the sexually mature trees were selected for the present study.

For DNA isolation young juvenile leaves soon after harvesting were placed in aluminium foil and sealed in a plastic bag which was appropriately labelled. Three samples from each population were placed in ice bucket and brought to lab for storage at -20°C until they were processed for DNA extraction.

Table 1. Details of Collection Sites for *Annona squamosa* Samples Used for Molecular Analysis

S. No.	Population Code	Name of Locality	Latitude	Longitude	Altitude
District: Udaipur					
1.	AS_1	Jhadol	24°21′ 32.95″N	73°31′ 53.17″E	2067 ft
2.	AS_2	Kotda	24°52′ 30.36″N	75°45′ 06.07″E	
3.	AS_3	Gogunda	24°45′ 02.25″N	73°32′ 04.28″E	2774 ft
4.	AS_4	Iswal	24°43′ 48.26″N	73°37′ 32.37″E	2406 ft
5.	AS_5	ManwaKheda	24°33′ 22.89″N	73°44′ 20.26″E	1838 ft
6.	AS_6	Madri	24°34′ 16.57″N	73°41′ 29.56″E	5202 ft
7.	AS_7	Kewre Ki Nal	24°31′ 11.95″N	73°39′ 45.22″E	
District: Chittaurgarh					
8.	AS_8	Badi Sadri	24°21′ 58.20″N	74°36′ 45.93″E	1564 ft
9.	AS_9	Chittaurgarh Fort	24°53′ 12.84″N	74°38′ 17.81″E	1342 ft
10.	AS_{10}	Mangalwar	24°35′ 32.63″N	74°17′ 44.48″E	1576 ft
11.	AS_{11}	Nimbahera	24°37′ 00.12″N	74°40′ 59.88″E	1447 ft
12.	AS_{12}	Kapasan	24°53′ 16.37″N	74°18′ 59.97″E	1488 ft
13.	AS_{13}	Rashmi	25°03′ 35.63″N	74°21′ 32.90″E	1536 ft
14.	AS_{14}	Gangrar	25°03′ 05.09″N	74°36′ 31.87″E	1435 ft
District: Rajsamand					
15.	AS_{15}	Kumbhalgarh	25°08′ 56.98″N	73°34′ 49.40″E	3466 ft
16.	AS_{16}	Tadgarh	25°41′ 26.49″N	73°58′ 21.78″E	2344 ft
17.	AS_{17}	Dewar Ki Nal	25°25′ 33.00″N	73°49′ 05.36″E	
18.	AS_{18}	Kankarwal			
19.	AS_{19}	Desuri Ki Nal	25°16′ 15.07″N	73°33′ 36.06″E	
20.	AS_{20}	Gomti Choraha	25°16′ 53.56″N	73°47′ 01.76″E	2189 ft
21.	AS_{21}	Devgarh	25°31′ 25.12″N	73°54′ 29.56″E	2084 ft

DNA Isolation and Purification

Total genomic DNA was extracted from the frozen leaf material using 4 standard protocols which comprised of Saghai-Maroof et al,[24] Dellaporta et al,[25] Storchova et al[26] and Gilmore et al.[27] However, DNA isolated from standard methods as well as from several modified methods was sticky and gelatinous having brown color. Such DNA samples when subjected to electrophoresis showed uneven migration producing smears. To overcome these problems a new and improved method was developed.

The young leaves (1 g) were deveined and grounded to a fine powder in a mortar and pestle using liquid nitrogen. The powder was transferred in 10 mL of CTAB buffer containing 100 mM Tris (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 2.5% CTAB (w/v), 1% PVP and 10 mMβ-mercaptoethanol (added freshly). It was mixed vigorously by vortexing and incubated at 60°C for 30 minutes followed by treatment with equal volume of chloroform: isoamyl alcohol (24: 1). The upper phase, obtained by centrifugation at 5125 X g for 15 minutes at room temperature was transferred to a fresh autoclaved centrifuge tube and then 1/10 volume of 3M sodium acetate (pH 5.2) and ½ volume of 5 M NaCl were added to it. DNA was precipitated using 0.6 volume chilled isopropanol and pelleted by centrifugation at 5125 X g for 10 minutes at 4°C. The supernatant was decanted and the DNA pellet was washed with 70% ethanol. Crude CTAB DNA pellet was air-dried and suspended in 500 μL of 0.5 mL high salt TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 1M NaCl) with brief heating at 65°C, if necessary. For some samples,

extra buffer was added to dissolve, as far as possible, the large carbohydrate-rich pellets. To each 1.0 mL of crude nucleic acid extract, 3 volumes of binding buffer (50 mM Tris (pH 7.5), 6M NaClO$_4$, 1 mM EDTA) was added and allowed to stand for 20 minutes. The mixture was centrifuged at 550 X g for 10 minutes and supernatant was transferred to a clean polypropylene centrifuge tube. To this 300 μL of diatomite suspension prepared as per the method described in following paragraphs, was added and contents were mixed for 20 minutes by regular and gentle inversion to allow the binding of DNA to the diatomite. The mixture was centrifuged at 550 X g for 10 minutes and supernatant was discarded. Then the diatomite was gently resuspended, in 1.5 mL of wash buffer I (3 volume binding buffer, 1 volume water), followed by centrifugation at 3000 X g for 15 seconds. The diatomite pellet was again resuspended in 1.5 mL of wash buffer II (1 volume 40 mM Tris (pH 8.0), 4 mM EDTA, 0.8 M NaCl, 1 volume ethanol) and centrifuged at 3000 X g for 15 seconds. The supernatant was decanted and the DNA diatomite pellet was air-dried and suspended in 300 mL TE buffer (10 mM Tris, pH 8.0, 1M EDTA) the tube was incubated with regular inversion at 11600 X g for 60 seconds and supernatant was collected into a clean 1.5 mL micro-centrifuge tube. The supernatant was precipitated by addition of 1M NaCl and 500 mL isopropanol and stored at −20°C for at least 2 hours, followed by centrifugation at 11600 X g for 5 minutes. The supernatant was discarded and the pellet was air dried. The pellet was re-dissolved in 100 mL 0.1 X TE buffer and stored at −20°C.

The purity of extracted genomic DNA was checked by digestion with restriction enzymes such as Eco RI (Bangalore Genei). Each reaction mixture of 30 μL volume contained 5 μg DNA, 3.0 μL 10X recommended buffer and 5U μL^{-1} restriction enzyme. The reaction mixture was incubated at 37°C for 10 hours. The digested DNA was electrophoresed on 0.8% agarose along with undigested DNA as a control. DNA concentration was measured using spectrophotometric method (U-2900 UV-Spectrophotometer, Hitachi, Japan).

Optimization of Polymerase Chain Reaction Conditions

For molecular characterization, various parameters of polymerase chain reaction (PCR), like concentration of genomic DNA, primers, dNTP, Taq polymerase, MgCl$_2$, annealing temperature and number of cycles were optimized using different concentrations.

Optimization of PCR Conditions for RAPD Primers

The primers were received as dry desalted powder and a stock solution of 100 pmol μL^{-1} was prepared. Desired amount of elution buffer was added to the powder and vials were left overnight at 4°C. Finally, a thermal shock at 65°C was provided for 10 min to ensure complete dissolution of primers. The stock solution was used to prepare the working solutions of 10 p mol μL^{-1}.

RAPD primers were evaluated for their applicability in PCR amplification of total genomic DNA of *A. squamosa*. For RAPD analysis 53 arbitrary decamer primers (RUF 201–RUF 220, Sigma Genosys, India and OP$_1$-OP$_{33}$,

Operon Technology, USA) were used for initial screening and only those primers were selected for the present study which provided clear and reproducible amplification products under similar conditions (Table 2).

(a) Annealing Temperature

Every individual primer was amplified in a range of annealing temperatures in order to generate maximum possible amplicons. For RAPDs, the annealing temperatures were varied in the range 32°C to 42°C.

(b) Effect of Magnesium Ion Concentration

Magnesium ions are essential for binding of Taq polymerase on to DNA template and therefore, their concentration in the reaction mixture is critical in determining the generation of amplicons. These ions are provided in the form of MgCl$_2$ in reaction mixture. MgCl$_2$ was varied in the concentration range of 1.5 to 3.5 mM at the interval of 0.5 mM and its effect on production of bands was studied.

(c) Effect of Concentration of dNTPs

In order to evaluate the effect of concentration of dNTPs on DNA amplification during PCR, the amount of dNTPs in the reaction mix was varied. Various concentrations viz. 0.10, 0.15, 0.2, 0.25 and 0.3 mM of dNTPs were tested. Other set of conditions was similar among all the variants.

(d) Effect of Number of Cycles

To observe the effect of number of repetitive cycles on DNA amplification, RAPD was performed with 35, 40 and 50 cycles.

(e) Effect of Concentration of DNA

To observe the effect of template DNA concentration on

Table 2. List of Screened Random Decamer Primers Used for Screening PCR Amplification of Total Genomic DNA in *Annona squamosa*

Primer	Sequence	Tm	Molecular Weight	No. of Monomorphic Bands	Band Size Range (bp)
RUF 202	TTGGCGGCCT	49.2	3034.90	5	300-1500
RUF 203	GGCGAAGGTT	38.1	3108.00	10	200-2000
RUF 206	ACGGGCCAGT	42.4	3053.00	5	400-1000
RUF 211	GGGTAACGCC	38.7	3053.00	6	300-1500
RUF 214	GAAGCGCGAT	40.0	3077.00	5	400-1000
RUF 216	CAGCGAACTA	26.2	3021.00	7	300-1500
RUF 217	CGACTCACAG	23.1	2997.00	5	700-1000
RUF 218	GGGCCTCTAT	31.7	3018.90	8	300-900
RUF 220	GGGTGAACCG	38.9	3093.00	6	300-1500
OP 01	TGCCGAGCTG	43.6	3044.01	8	300-1500
OP 03	ACCCCCGAAG	43.6	2981.95	8	300-1500
OP 05	TTCGAGCCAG	39.5	3028.01	6	400-1500
OP 07	AAAGCTGCGG	39.5	3077.04	7	300-1000
OP 09	CTACGGAGGA	39.5	3077.04	9	300-1500
OP 10	GGCACTGAGG	43.6	3093.04	8	400-1500
OP 11	TCACGTCCAC	39.5	2947.95	7	300-1000
OP 12	CTGACGTCAC	39.5	2987.98	5	400-1000
OP 13	TGCCCGTCGT	43.6	2994.98	9	300-2000
OP 17	CCGCCCAAAC	43.6	2941.92	5	400-4000
OP 20	GAGCGTCGAA	39.5	3077.04	6	400-2000
OP 21	CCCAGCTGTG	43.6	3003.98	9	300-1000
OP 22	CACAGGCGGA	43.6	3062.01	8	300-2000
OP 24	AGCAGGTGGA	39.5	3117.07	9	400-1000

PCR amplification 20, 30, 40 and 50 ng DNA were used separately to generate amplification products. All these concentrations of DNA were amplified with 2 primers in independent reactions at their respective annealing temperatures. All other conditions were kept constant.

(f) Taq Polymerase
Thermostable Taq DNA polymerase (Bangalore Genei, India) purified from *Escherichia coli* expressing a cloned *Thermus aquaticus* DNA polymerase was used in all amplifications. This enzyme has both 5' → 3' DNA polymerase and 5' → 3' exonuclease activity but lacks a 3' → 5' exonuclease activity. The enzyme consists of a single polypeptide with molecular weight of approximately 94 kDa.

In order to evaluate the effect of concentration of Taq DNA polymerase on DNA amplification, five concentrations viz. 0.5, 0.75, 1.0, 1.25 and 1.5U were tested.

Optimization of PCR Conditions for ISSR Primers
For ISSR, primers were evaluated for their applicability in PCR amplification of total genomic DNA of *A. squamosa*. A total of 48 ISSR primers (16-31mer) (Sigma Genosys, India) were used for initial screening at different annealing temperatures.

(a) Annealing Temperature
Every individual primer was amplified under a range of annealing temperatures in order to generate maximum possible amplicons. For ISSRs, the annealing temperatures were varied in the range 40-60°C (Table 3).

Data Scoring and Statistical Analysis
Data Scoring
Data analysis of digital images was done by the Gene Tool Image Analysis Software provided with Gel Documentation System (Syngene, UK). It permits fast and consistent analysis of electrophoresed gel images performing precise band edge detection. Amplification products were scored from the gel images as presence or absence of bands. Each band was treated as one marker. Homology of bands was based on the distance of migration of amplified DNA fragments according to their molecular weights in the gel. The presence of band was scored as '1' and absence of band as '0'. All the reactions were repeated twice and only those bands which were reproducible for all the runs were considered for the analysis. Co-migrating bands were considered to represent the same locus and thus as the same band while scoring.

Two binary qualitative data matrices of the different RAPD and ISSR molecular phenotypes were constructed, where row corresponded to the populations and the column to the bp ranges. These 2 matrices were then used for the statistical analysis.

Statistical Analysis
The binary data matrices of both ISSR and RAPD were analysed via SIMQUAL of the NTSYS-pc version 2.1.[28] This converts the binary matrices into similarity matrices using the Nei and Li index which is also known as Dice's coefficient. It is represented as D = 2 nab/ (na + nb), where *na* and *nb* represent the number of bands present in lanes a and b, respectively and nab represents the number of bands shared by both lanes. *D* can acquire any value between 0 and 1, where 0 means no bands in common and 1 means patterns are identical. This index takes only positive matches into account and placing a weight of 2 on shared bands, purportedly permits a better differentiation of individuals with low level of similarity. The similarity matrices serve as a starting point for subsequent multivariate analyses. The main purpose of multivariate

Table 3. List of ISSR Primers Used for Screening PCR Amplification of Total Genomic DNA in *Annona squamosa*

Primer	Sequence	Tm	Molecular Weight	No. of Monomorphic Bands	Band Size Range (bp)
IS 30	(AGAG) 4C	46.8	5366	7	500-1500
IS 32	(GAGA) 4T	42.9	5381	7	300-1500
IS 38	(CACA) 4T	51.1	5061	4	400-1000
IS 45	(TCTC) 4C	47.5	4974	7	300-2000
IS48	(ACAC)4C	53.3	5046	5	400-1000
IS49	(ACAC)4G	54.9	5086	5	400-1000
IS56	(AGAG)4C+TT	45.6	5678	9	300-1500
IS57	(AGAG)4C+TC	45.6	5663	8	300-1500
IS63	(GAGA)4C+TC	46.0	5663	5	300-1500
IS64	(GAGA)4C+TG	47.2	5703	3	200-600
IS76	(TCTC)4A+GG	48	5335	4	300-1500
IS78	(ACAC)4C+TA	49.8	5367	9	300-1500
IS86	(GATG)4AT	51	5618	6	300-1000
IS90	(GAA)5A	47.8	5672	5	400-2000
IS102	(GAGA)3G	49	4780	6	400-1500
IS 106	(AGAG)3(A+T+C)2G+TC	41.9	5347	5	300-800
IS108	(CTCT)3(G+A+C)2G+AT	44.2	5028	4	400-1000
IS 111	(ACAC)3(G+A+T)2G+TC	47	5093	4	400-1000

statistics is to condense the differences between the entries of many characters into fewer characters and to visualize these entries in a multi-dimensional space.

Population Genetic Analysis

The data matrix of RAPD and ISSR phenotype was analyzed using POPGENE (version 1.31) software[29] to assess genetic diversity and gene differentiation. The parameters used are listed as follows; the percentage of polymorphic bands (PPB), Nei's gene diversity (H_E), coefficient of genetic differentiation (GST), gene flow (N_m), observed number of allele per locus (N_a) effective number of allele per locus (N_e) and Shanon's index of phenotypic diversity (I).[30] Cluster analysis, which is the most used type of classification analysis, that groups similar objects into identifiable and interpretable classes that can be distinguished from neighbouring classes and to resume these relationships in a dendrogram (tree-diagram). In the present study dendrograms were constructed using the unweighted pair-group method of arithmetic average (UPGMA). A principal co-ordinate analysis was performed in the study using EIGEN program of NTSYS-pc version 2.2.[31] Mantel test was also performed to estimate a correlation between the matrices of Neis[32] genetic distances using NTSYS-pc version 2.1 (1000 permutations).[28]

Results

Field Surveys and Sample Collection

Field surveys were made in the adjoining areas of Udaipur, Chittaurgarh and Rajsamand district and a total of twenty one populations were collected from different locations. Three trees were identified in each area and leaf samples were collected accordingly.

DNA Isolation and Purification

Leaf samples were subjected for DNA isolation using 4 methods. Of all the methods tried the modified Gilmore's method was found to be the best and was set as a standard protocol for DNA isolation during all subsequent analyses. DNA loss through this method was minimal. The DNA obtained from all the accessions were of good quality yielding a ratio between the ranges of 1.65 to 1.85 as read spectrophotometrically on a double beam UV spectrophotometer. The total DNA yield varied from 200-600 ng/μL. Both the quality and yield of DNA was satisfactory to be used for primer screening and carrying out PCR reactions. DNA samples when digested with the restriction endonuclease Eco RI showed complete digestion under standard condition. Genomic DNA was extracted from this new improved method using cetyltrimethylammonium bromide (CTAB) with purification based on diatomite.[33]

Optimized PCR Conditions

Optimized Conditions for RAPD Analysis

Of the 53 RAPD primers initially screened, 19 produced clear and scorable amplification products (Figure 4). These primers yielded a total of 148 amplicons with an average of 8 bands per primer. The amplified fragments ranged from 5 kb to 0.2 kb.

RAPD profiles were produced through PCR amplification using the protocol described by Williams et al.[8] with minor modifications. All the PCR reactions were carried out (under optimized conditions) in 0.2 mL polypropylene PCR tubes (Bangalore Genei, India) using Thermal Cycler (Eppendorf). Each 20 μL reaction mixture contained 1 X Taq buffer (100 mM Tris-Cl (pH 9.0), 500 mM KCl, 1.5 mM $MgCl_2$ and 0.1% Gelatin, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix (Bangalore Genei, India), 20 pmol oligonucleotide primers (Sigma Genosys, India and Operon Technologies, USA), 1U Taq DNA polymerase (Bangalore Genei, India) and 40 ng template DNA Fig 5-10). All reactions were subjected to initial denaturation at 94°C for 4 minutes followed by 40 amplification cycles, each consisting of 1 min at 94°C (denaturation step), 1 minute at 37°C (annealing step) and 2 min at 72°C (extension step) with a final extension of 7 minutes at 72°C. In order to evaluate the effect of different components on DNA amplification, only one component was varied at a time and other conditions were kept constant as described above.

Optimized Conditions for ISSR Analysis

Out of 48 only 15 primers yielded DNA profiles on 1.5% agarose gels. A total of 103 loci were scored across all the populations from individual primer with an average of 5.7 bands per primer (Figure 5). The approximate size of the
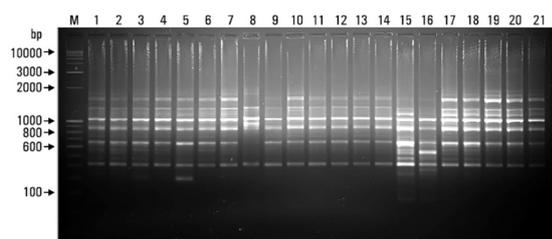

Figure 4. RAPD Amplification of Total Genomic DNA Samples of 21 Populations of *Annona squamosa* Belonging to 3 Districts of South East Rajasthan OP-20.
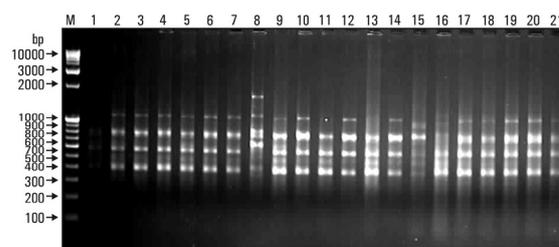

Figure 5. ISSR Amplification of Total Genomic DNA Samples of 21 Populations of *Annona squamosa* Belonging to 3 Districts of South East Rajasthan IS-48.

largest fragment produced was approximately 2 kb and the smallest easily recognizable fragment produced was approximately 0.2 kb.

For ISSR analysis 20 µL reaction mixture contained 1 X Taq buffer (100 mM Tris-Cl (pH 9.0), 500 mM KCl, 15 mM $MgCl_2$ and 0.1% Gelatin), 2.5 mM $MgCl_2$, 0.0 mM dNTPs (Bangalore Genei, India), 20 p mol oligonucleotide primers (Sigma Genosys, India), 1U Taq DNA polymerase (Bangalore Genei, India) and 40 ng template DNA. Thermal cycler was programmed with an initial denaturation at 94°C for 5 min, followed by 40 cycles of 30 seconds denaturation at 94°C, 45 seconds annealing at respective annealing temperatures (45-60°C) and 90 seconds extensions at 72°C. A final 10 minutes hold at 72°C ensured full extension of all amplification products. The amplification products were stored at 4°C. In order to evaluate the effect of different annealing temperatures on DNA amplification using single primer or pair combination, only one component was varied at a time keeping other conditions constant as described above.

Population Genetic Analysis
Genetic Diversity Within Samples of a Population
RAPD and ISSR analysis were performed to detect intra-population variability. Variability was assessed within the population of individual sites using both the primers where minor variability in terms of band intensity was observed in a few instances. Three samples collected from the plants within a locality (population) produced almost monomorphic bands while employing 19 RAPD and 15 ISSR primers.

Genetic Diversity Among and Within Populations of Groups
Population Structure
Various genetic diversity parameters at the species level were estimated using POPGENE software. At the species level the observed number of alleles per locus (Na) was 1.7391 and the value of effective number of alleles per locus (Ne) was 1.4188 (Table 4). Averaged across all populations the percentages of polymorphic loci were 50.93%, 47.95% and 29.19% for Udaipur, Chittaurgarh and Rajsamand populations, respectively. The average observed number of alleles per locus (Na) was 1.3602 (ranging from 1.2795 to 1.5093) and Ne was 1.240 ranging

from (1.1733 to 1.3528) (Table 4). Among the populations, the Udaipur district exhibited high level of variability PPB = 50.93%, Na = 1.5093, Ne = 1.3528, H_E = 0.28710 .The average gene diversity was estimated to be 0.1360 (Hs) within the populations ranging from 0.1002 to 0.1964. Considering Hardy Weinberg equilibrium the total Nei genetic diversity (H_T) at the species level was 0.2509 (Table 5). The overall level of inferred gene flow (N_m) was estimated as 0.5914 individuals per generation among populations. Genetic differentiation (G_{ST}) was estimated as 0.4581 indicated that 45.8% of genetic variability is distributed among populations.

The Shannon's index was 0.2002 (Hpop) and 0.3800 (H_{sp}) at population and species level, respectively (Table 6). The proportion of genetic variation within population (H_{pop}/H_{sp}) and proportion of genetic variation among populations ($H_{sp} – H_{pop}$)/ H_{sp} yielded 0.52 and 0.473 values, respectively.

Table 5. Measures of Genetic Diversity (Nei, 1973) Under the Assumption of Hardy-Weinberg (fis=0)

Parameter	Value
H_T	0.2509
H_S	0.1360
D_{ST}	0.1149
G_{ST}	0.4581
N_m	0.5914

H_T = Total gene diversity, H_S = Gene diversity within population, D_{ST} = Gene diversity among population, G_{ST} = Coefficient of gene differentiation, N_m = Nei's gene flow.

Table 6. Partitioning of Genetic Variation Into Within Populations Based on Shannon's Information Index

Parameter	Mean
H_{pop} [a]	0.2002
H_{sp} [b]	0.3806
H_{pop}/H_{sp} [c]	0.52
$\left(\dfrac{H_{sp} - H_{pop}}{H_{sp}}\right)$ [d]	0.473

[a] Mean genetic variation for the populations.
[b] Mean genetic variation of the total populations.
[c] Proportion of genetic variation within population.
[d] Proportion of genetic variation between populations.

Table 4. Measures of Genetic Diversity in 3 Districts (Representing 21 Populations) of *Annona squamosa* Assuming Hardy-Weinberg Equilibrium (fis=0) Based on Cumulative Data (RAPD + ISSR)

District	Polymorphic loci (PPB)		Observed Number of alleles Na ± SD	Effective Number of Alleles Ne ± SD	Neigene Diversity HE ± SD	Shanon Index I ± SD
	No.	%				
Udaipur	82	50.93	1.5093 ± 0.5015	1.3528 ± 4091	0.1964 ± 0.2157	0.2871 ± 0.3047
Chittaurgarh	45	47.95	1.2795 ± 0.4502	1.1733 ± 0.3219	0.1002 ± 0.1756	0.1494 ± 0.2540
Rajsamand	47	29.19	1.2919 ± 0.4561	1.1950 ± 0.3407	0.1113 ± 0.1848	0.1641 ± 0.2663
Mean	58	42.69	1.3602	1.240	0.1360	0.2002
Overall	119	73.91	1.7391 ± 0.4405	1.4188 ± 0.3520	0.2509 ± 0.1829	0.3800 ± 0.2577

Cluster Analysis

RAPD and ISSR data were used to make pair wise comparison and unique amplification products to generate a similarity matrix with NTSYS (version 2.1) software.

(a) RAPD

Similarity values for all the 21 populations ranged from 0.6 to 0.94%. The similarity matrix presenting Dice's similarity coefficient was used to cluster the data following the UPGMA analysis NTSYS-PC (version 2.1). The resultant dendrogram grouped 20 of the 21 populations into 4 distinct clusters A, B, C and D. The populations collected from As_1 to A_7 are grouped in same cluster. Similarly, As_9 to As_{14} are grouped in same cluster. It was observed that populations collected from same geographical and ecological zone are more similar to each other and grouped in one cluster. However, populations As_8 collected from Badi Sadri remained unclustered and did not show similarity with any of the groups.

(b) ISSR Analysis

The genetic distance with ISSR data set using Dice coefficient of similarity ranged from 0.61 to 0.986. The maximum genetic similarity was observed between As_6; As_7 and As_{20}; As_{21} populations. The minimum genetic similarity of 0.61 was observed between As_3 and As_{15}. The UPGMA tree consisted of 2 major clusters. Cluster I grouped together all the Udaipur populations (As_1–As_7) whereas cluster II consisted of populations of Chittaurgarh and Rajsamand district. Cluster II is further subdivided into 2 subclusters (II A and II B). The cluster II A consisted of all the populations of all the populations of Chittaurgarh district and cluster II B grouped Rajsamand populations.

(c) Combined Tree

The similarity value for all the 21 populations based on combined data ranged from 0.67 to 0.92 (Table 7). The accessions pairs (As_1; As_2) and (As_{19}; As_{21}) showed maximum genetic similarity value. The minimum genetic similarity was observed between As_1; As_8; As_2: As_8; As_6: As_8 and As_8: As_{19}) populations pairs (Figure 6). The UPGMA tree grouped the populations into 2 cluster. The populations of different districts grouped into a single cluster and did not group with clusters, represents different groups. The dendrogram deprived an apparent correlation between geographical distance and combined molecular data.

Principle Component Analysis

(a) PCA Based on RAPD Data

The genetic relationships among the 21 populations obtained from principle component analysis (PCA) showed almost similar grouping patterns with dendrogram. In 3-D plot of PCA, population As_8 was the most diverse as it was most distinctly separated in the plot. The PCA plot grouped the populations into 3 different clusters. Populations As_{15} and As_{16} of Rajsamand district clustered with populations of Udaipur district. The populations of Chittaurgarh district remain separated and did not cluster with other groups.

(b) PCA Based on ISSR Data

The PCA plot based on ISSR data grouped the populations into clusters. In the 3-D plot of ISSR, there was considerable overlapping between the samples from different populations; still some patterns could be discerned. The As_4 and As_9 populations did not clustered with any other groups, and aligned at the edges of the plot. The populations of Udaipur district formed a separate group, while the populations of Rajsamand and Chittaurgarh district clustered into a single group.

(c) PCA Based on Combined Data

PCA of cumulative data set also supported the clustering pattern of the populations. The populations As_4 and As_5 of Udaipur district did not grouped in any cluster (Figure 7). There was considerable overlapping between the populations.

Mantel's Test

The Mantel Z-statistic is used for comparison of different fingerprinting methods and to correlate the respective distance matrices. The test carried out between three datasets revealed that combined data versus RAPD had the maximum correlation coefficient (0.92) in comparison to cumulative combined ISSR (0.91). Among the individual DNA fingerprinting methods RAPD Vs ISSR showed the value of (0.72). The values revealed that RAPD and cumulative data have good correlation and are best fit to each other. The mantel between the matrices of RAPD and ISSR based on Nei and Li similarity index gave r = 0.72 after 1000 random permutations revealed a low correlation between these 2 datasets (Table 8).

Discussion

The study of genetic diversity of tropical tree species is useful to landscape management, plant genetic resource inventory and biological conservation. Conservation programs are already using data generated by molecular techniques to evaluate the genetic structure and diversity of natural populations.[4,33]

In present study 35 primers (19RAPD +15ISSR) primers produced 73.9% polymorphism. Similar results with 73% polymorphism were reported by Bharad et al[35] by analyzing 11 genotypes of *A. squamosa* using five RAPD primers. Avery high level of polymorphism of 90% and 87.8% have been reported in *Annona muricata* L.[36] and *A. crassiflora*[5] respectively. In contrast, a very low polymorphism of 29% was observed by using 20RAPD primers among 64 accessions of *A. squamosa*.[37] A study using 20 RAPD and 30 ISSR primers revealed a moderate level of polymorphism of 58% in 4 *Annona* species of Andaman Island[38] Thus, the per cent polymorphism may vary significantly in similar or different plant species. This is explicable as the product amplification depends upon the sequence of random

Table 7. Dice's Similarity Coefficient Based on Combined Pooled Over 19 Primers in 21 *Annona squamosa* Populations

|	AS1	AS2	AS3	AS4	AS5	AS6	AS7	AS8	AS9	AS10	AS11	AS12	AS13	AS14	AS15	AS16	AS17	AS18	AS19	AS20	AS21
AS1	1.000																				
AS2	0.950	1.000																			
AS3	0.894	0.906	1.000																		
AS4	0.881	0.894	0.888	1.000																	
AS5	0.832	0.857	0.863	0.900	1.000																
AS6	0.888	0.863	0.881	0.906	0.881	1.000															
AS7	0.832	0.832	0.863	0.863	0.850	0.931	1.000														
AS8	0.670	0.670	0.689	0.689	0.677	0.670	0.689	1.000													
AS9	0.683	0.695	0.714	0.714	0.726	0.683	0.714	0.838	1.000												
AS10	0.708	0.708	0.751	0.726	0.714	0.695	0.751	0.838	0.900	1.000											
AS11	0.701	0.714	0.720	0.757	0.757	0.714	0.732	0.844	0.881	0.919	1.000										
AS12	0.695	0.695	0.739	0.751	0.763	0.720	0.726	0.813	0.900	0.925	0.919	1.000									
AS13	0.701	0.726	0.708	0.745	0.770	0.701	0.708	0.832	0.881	0.869	0.900	0.894	1.000								
AS14	0.714	0.739	0.732	0.745	0.782	0.701	0.720	0.782	0.866	0.881	0.900	0.944	0.925	1.00							
AS15	0.695	0.708	0.689	0.726	0.739	0.695	0.664	0.701	0.714	0.689	0.720	0.726	0.770	0.745	1.000						
AS16	0.720	0.708	0.714	0.739	0.739	0.708	0.701	0.726	0.776	0.751	0.732	0.763	0.795	0.795	0.863	1.00					
AS17	0.726	0.739	0.757	0.791	0.807	0.751	0.732	0.683	0.732	0.732	0.751	0.770	0.788	0.801	0.819	0.869	1.000				
AS18	0.684	0.702	0.720	0.757	0.770	0.726	0.708	0.683	0.708	0.720	0.751	0.757	0.776	0.788	0.832	0.844	0.925	1.00			
AS19	0.714	0.726	0.745	0.782	0.782	0.751	0.732	0.670	0.720	0.745	0.726	0.770	0.763	0.788	0.795	0.844	0.888	0.900	1.000		
AS20	0.726	0.726	0.745	0.782	0.795	0.751	0.720	0.695	0.720	0.720	0.726	0.757	0.776	0.788	0.807	0.857	0.913	0.435	0.900	1.000	
AS21	0.739	0.751	0.732	0.807	0.795	0.751	0.732	0.695	0.720	0.745	0.751	0.770	0.788	0.801	0.807	0.844	0.900	0.925	0.950	0.937	1.000
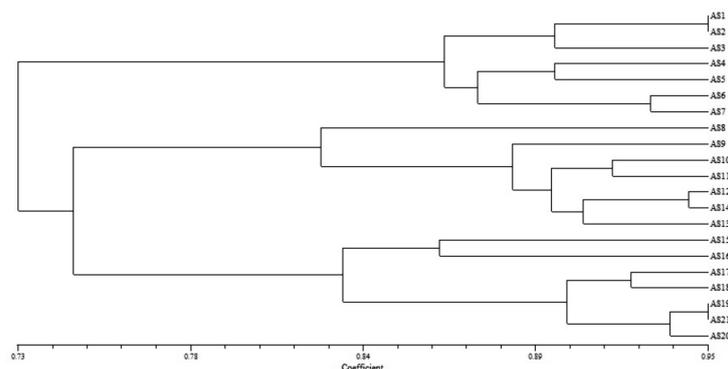
Figure 6. Dendrogram Constructed by UPGMA Clustering Among 21 Populations of *Annona squamosa* From 3 Districts of Rajasthan. Combined (RAPD+ISSR).
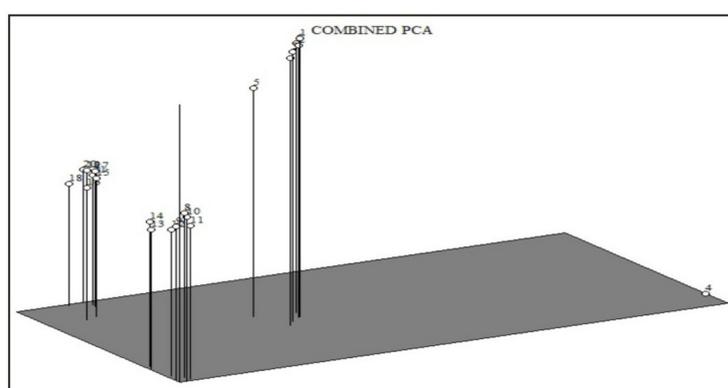

Figure 7. Inter-individual Relationships Among 21 Populations of *Annona squamosa* in Multidimensional Space as Estimated by PCA Analysis. Combined (RAPD+ISSR).

primers and their compatibility with genomic DNA.[39] suggest that greater genetic variability can be achieved by collecting few individuals from different locations rather than larger number of individuals from the same location. Furthermore, Poerba and Martani,[40] suggest that DNA band resulted by each primer is different in both size and amount of DNA bands.

The population genetic analyses of the cumulative data set revealed low levels of genetic diversity (H_{pop}= 0.2002) within populations of *A. squamosa.* The present result was in agreement with *A. muricata* species.[35] The existence of low genetic diversity within *Annona* species is attributed to self pollination.[4,41] Although *A. squamosa* flowers are adapted to cross pollination, but sometimes in a period from 36 to 48 hours both sexual organs ripe

simultaneously facilitating self pollination. A possible cause of such variation may be that *A. squamosa* is reported to be a mixed system crossing i.e. plant may intersect inbreeding.[42] Although dichogamy and low population density of pollinator insect are important limiting factor to successful natural pollination, the effect of climate and pollen variability seem to interfere greatly.[43] Further, Nybom[9] explained that seed dispersal did not have significant effect and taxa where seeds are dispersed by insects or by attaching to animals had lower values than taxa that use potentially more efficient agents, i.e. wind or water. This may be a possible reason for *A. squamosa* populations having low gene diversity within the populations.

Analysis of molecular data using statistics demonstrated significant differentiation among populations. The value of genetic differentiation detected among populations of *A. squamosa* G_{ST} = 0.458) was higher than average for outcrossing plant species (G_{ST} = 0.22).[9] The highly significant genetic differentiation found among populations was very unusual for outcrossing and insect pollinated species.[44] This may be mainly due to limited gene flow and genetic drift. As the populations are

Table 8. Mantel Correlation Between the Genetic Distances Obtained From RAPD, ISSR and Cumulative Data Analysis Among the *Annona squamosa* Populations

Marker Pair	RAPD vs ISSR	RAPD vs Cumulative	ISSR vs Cumulative
r	0.72701	0.92930	0.91816
P	1.000	1.0000	1.0000

restricted to distant mountains with intervening plains and hill region which may also reduce gene exchange between populations. In addition low estimates of gene flow among populations of *A. squamosa* (N_m = 0.59) indicated that the range of insect-mediated pollen dispersal might not be as far as generally expected. Similar genetic structure with above-average values of population genetic differentiation was detected in *Taxus fuana* Nan Li and R.R. Mill,[45] *Terminalia bellerica* Gaertn. Roxb.[46] and *Boswellia serrata* Roxb. ex Colebr[47] Contrary to present observations high value of N_m was observed among populations of *Torreya jackii* Chun[48] (G_{ST} = 0.63,) and *Omphalogramma souliei* Franch[49] (G_{ST} = 0.60).

In present study a low level of genetic diversity within population and high genetic diversity among populations could be attributed to several factors such as population fragmentation, breeding system, restricted geographical distribution and microclimate.[50]

The UPGMA dendrogram constructed using the combined data of the 2 sets of molecular markers was almost similar to those obtained separately with each marker system. The highly structured populations were of Rajsamand district. The grouping of Chittaurgarh and Udaipur populations probably indicated that they have some common ancestry. The dendrogram derived an apparent correlation between geographical distance and combined molecular data. Similar to present study a significant correlation between different dendrograms obtained from various molecular markers has been reported.[34,35,51,52] However, Suratman et al,[36] in *A. muricata* reported that dendrogram did not indicate geographical origins but showed only genetic similarity. One of the main applications of these clusters is the estimation of the genetic distance among populations and to establish effective conservation practices.

In present study results have demonstrated that the both ISSR and RAPD markers are suitable for characterization and assessment of genetic diversity in *A. squamosa*. The information about genetic diversity will be helpful to avoid any possibility of elite germplasm becoming genetically uniform.[53] The data provides an opportunity for improvement and genetic conservation program of tree as well as in making rational base decision regarding prioritising population which require conservation.

Conclusion
In conclusion, results have shown that the RAPD and ISSR markers showed low level of genetic diversity within populations of districts and showed significant genetic differentiation among populations of *A. squamosa*. The distribution of genetic variation among *A. squamosa* populations was unusual for this out crossing long-lived species, but it may be due to narrow range of populations that was studied during present investigation. Further, studies including collection of samples from entire range

of this species from across wide geographic across of the country may help to fully understand the genetic structure of *A. squamosa* populations.

The present study has made a useful contribution towards a better understanding of genetic variation and population structure of *A. squamosa* in Aravallis hill region helping formulation of efficient programs for the presentation of genetic diversity.

Competing Interests
None.

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