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Diversity Assessment of *Annona muricata* L. Through Molecular Characterization using SSR Markers

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***Abstract*— *Annona muricata* L. (Magnoliales: Annonaceae) is a tropical fruit tree with a long history of traditional use. The object of this research was to determine the genetic diversity of the *Annona muricata* L. species using SSR markers. Thirty-four accessions of *A.muricata* leave samples and a leaf sample of *A.cherimola* as a control were collected from different locations in Sri Lanka. Genetic diversity of *A.muricata* was evaluated using eight microsatellites markers developed in *Annona cherimola*. Optimum annealing temperature of each primer was selected after the gradient PCR. Total nine genotypes were detected with 1.125 alleles per locus. The low levels of mean observed and expected heterozygosity ranged from 0.0147 and from 0.0140, respectively. The average Polymorphic Information Content (PIC) value for all markers used was 0.0131, the highest PIC value 0.1046 was obtained for the primer LMCH9.The high level of PIC value and polymorphism indicate that the primer LMCH9 is well suited for diversity assessment of *Annona muricata* germplasm. Accessions 168, 25, 82 & 49 were gave heterozygous alleles band for primer LMCH9. As a result, it was revealed a relatively limited genetic diversity within selected Annona accessions with respect to *Annona cherimola* markers.**

***Index Terms*— *Annona muricata*, SSR markers, Microsatellites, PIC value and Polymorphism.**

I. INTRODUCTION

Annona muricata L. is the family of Annonaceae (commonly known as Soursop or Katuanoda) with about more than 130 genera and 2300 to 2500 species are available, it's belongs to the genus: Annona, species: muricata. This is mostly found in home gardens. Only a limited number of researches has been conducted on Annona species in Sri Lanka (8). The major growing area in Sri Lanka for *A.muricata* is the wet and intermediate zone of the island (3). The *A.muricata* herbal properties and actions are kill cancer cells, slows tumor growth, diabetes, asthma, kills bacteria, kills parasites, reduce blood pressure, reduce fever, stimulate digestion and kills viruses (1). Most of the research on graviola focuses on a novel set of photochemical called Annonaceous acetogenins(9). Fruits which can kill the tumor cells more effectively in comparison to chemotherapy without producing harming normal cells (6). The use of molecular markers in tropical fruit tree breeding is greatly assisting in solving a number of difficult challenges for breeders such as the development of complex family structures for recombination mapping and for recurrent selection (7). *Annona muricata* has 6 chromosomes. Therefore, this study is aimed to analyze the diversity of *Annona muricata* using sixteen SSR markers for samples collected from the different geographical region.

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II. MATERIALS AND METHODS

A. Sample collection

Total number of 35 samples were collected for this experiment. Leaves of selected *Annona muricata* accessions were collected from Fruit Crops Research and Development Station, Gannoruwa and home garden in Killinochchi and Mullaitivu. One leaf samples of *Annona cherimola* was collected for control from Fruit Crops Research and Development Station, Gannoruwa. Extracted genomic DNA stored at -180C was used from Plant Genetic Resource Center that leaf samples were collected from Agricultural Research Station (ARS), Girandurukotte.

Accession No of Leaf Samples: Killinochchi- K1, Mullaitivu - M2 & M3, Fruit Crops Research and Development Station, Gannoruwa- 41, 235, R2, Fruit Crops Research and Development Station, Gannoruwa- CH, Agricultural Research Station ARS, Girandurukotte - 99, 18, 149, 181, 82, 177, 170, 74, 15, 66, 65, 103, 91, 172, 25, 130, 92, 183, 43, 108, 104, 49, 167, 122, 86, 01, 58 & 125.

B. Genomic DNA extraction

Genomic DNA was isolated from frozen leaf samples by using modified CTAB method.2g of frozen leaf sample was used in DNA extraction fallowing the protocol of D.D.P.B.D.Dehigaspitiyaet al.,(2015).

DNA extraction protocol: 2g of newly expanded leaves samples were cut in to small pieces and grinded with liquid nitrogen into a powder form used by pestle and mortar. Grinding was repeated until the leaf pieces were sufficiently macerated. Transfer the grinded leaf samples into a centrifuge tube containing 4 ml preheated (65°c) 2% CTAB (CTAB-2.0g, 5M NaCl- 28.0ml, 0.5 M EDTA- 4.0ml, 1M Tris HCl- 10.0ml and Distilled water up to 100.0ml) buffer. 10 µl of 0.2% β-mercapto ethanol was added into the centrifuge tube under the lamina floor. The content was incubated in water bath at 65°c for 30 min. 4 ml of Isoamyl alcohol: Chloroform (1:24) mixture was added into each centrifuge tube. The content was mixed gently for 20 min used by electrical shaker. Then the content was centrifuge at 10000 rpm for 15 min. After the centrifugation, the upper aqueous layer was transferred into another centrifuge tube. 0.4 ml of Isoamyl alcohol: Chloroform (1:24) was added again into centrifuge tube. The content was mixed gently for

10 min used by electrical shaker. Then the content was centrifuged at 1000 rpm for 15 min. Upper aqueous solution was carefully separated by micropipette into small vial. 2/3 rd of ice cold isopropanol 3ml was added, invert tubes slowly and mixed well. The DNA was spool out by tip of micropipted.100µl of DNA washing alcohol (70%) was added to wash the extracted DNA. Pellets obtained by centrifugation at 1000 rpm for 15 min and pipetted out the supernatant and allowed to air dry under room temperature. Completely dried pellets were resuspended in TE buffer according to the amount of pellets and kept at room temperature to dissolve the pellets. DNA was stored at -18 °c until used for PCR.

C. *Analysis of raw DNA*

The concentration of DNA in a sample and its condition are often estimated by running the sample on an agarose gel (10) We were used 0.8 % Agarose gel (0.24g of agarose/30 ml of 0.5X TBE buffer) to check whether the DNA is degraded or not.2µl of raw DNA was loaded into the wells with loading dye used by a micropipette, last well was loaded with 2µl of λ_{25} ladder 25ng/µl. The gel was run at 55V (constant voltage) for 60 minutes. The gel was strained in EtBr in a dark room for 20 min and washed by distilled water for 15 min. Finally, gel image was taken under the UV light.

D. *Dilution of raw DNA and DNA quantification*

Isolated raw DNA was dissolved in TB buffer based on the concentration of DNA in extracted raw DNA. Raw DNA was diluted to about 25ng/ µl. Diluted DNA was loaded on a 0.8 % agarose gel mixed with orange G dye. One well was loaded with λ_{25} molecular nucleotide. Buffer tank was connected to 55V power supply and allowed to run for 45 min. After the stained, gel image was taken under the UV light. DNA concentration was estimated by visually comparing band intensities of the extracted DNA with the molecular ladder of known concentration of λ_{25}.

E. *Polymerase Chain Reaction*

PCR reaction was performed in 10 µl volume using Takara and Veriti thermocyclers. The reaction mixture was contained 2 mM $MgCl_2$, 0.25 mM of each dNTP, 1µl of each primer (forward and reverse), 0.5 unit of Taq DNA polymerase and 40ng of genomic DNA. Temperature regime consisted of an initial denaturation step of DNA at 94°C for 1 min, followed by 35 cycles: denaturation at 94°C for 30 secs, annealing from 50°C to 57°C (specific for each primer pair) for 30 sec and extension at 72°C for 1 min after the final cycle samples were incubated at 72°C for 5 min to ensure the complete extension. The primers were used for PCR amplification showed in Table 1.

Table 1: SSR markers used for PCR

Primer name		Sequence	Repeat motif	Product size (bp)
LMCH 1	F-	CTCTTCAAAGGTACGACTTC	(CT)20	291 -312
	R-	TTGAGAAAAGGATAAGGATT		
LMCH 2	F-	CATTAACAGAGCATCAAAAT	(CCT)5	166- 170
	R-	AGATTGAGAAGTCGTACCTT		
LMCH 3	F-	TCTGTGAAAATACTCTCGTA	(GA)13	225-248
	R-	TCTCCACTGAATAATCTTTAAT		
LMCH 4	F-	ATTAGAACAAGGACGAGAAT	(GA) 14	112-128
	R-	CCTGTGTCTTTCATGGAC		
LMCH 5	F-	CCCACTCTTCTACCCTCAAC	(CT)10	155- 160
	R-	CAAGTCCCTGTAAGAATCAGA		
LMCH 6	F-	GGCATCCTATNITCAGGTTT	(CT)14	220-254
	R-	TTAAACAT TT TGGACAGACC		
LMCH 7	F-	ATCACCAACACTGAATCT TA	(GA)9	206-212
	R-	AATTTTTACCTGTAGACGTG		
LMCH 8	F-	AATTACGCAGATCACAGTAGC	(GA)8	247-251
	R-	CATCTTGCCTTGCTCTCTAC		
LMCH 9	F-	TCAAACACGTATAGAAAACC	(GA)6	170-172
	R-	TATGTGAAAGATCAAAAAGAG		
LMCH 10	F-	TTCT TGTTGGGAAGTATAGA	(CT) 12	220-264
	R-	GAAATCAATGTAGGTGTGAC		
LMCH 11	F-	TACCTCTCGCTTCTCTTCCT	(CT) 10	173- 176
	R-	GATGATTAGACACAAGTGGATG		

LMCH 13	F-	ATACGACTAGCGGAGCAGAC	(GA)20	304-334
	R-	GAGAATGTCGAGGGAGATGT		
LMCH 16	F-	TGAAAAATAACAAGAATGTAA	(GA)11	216-230
	R-	GGATAAACAAAGCAGTAAATC		
LMCH 29	F-	GTACCATCTTTTAGGAAATC	(GA)9	185-195
	R-	TGCAATCTATGTTAGTCAC		
LMCH 33	F-	AAGAAATGGGAGTAAATAGTG	(CT)11	242-249
	R-	ACGGTTGTGAATAGTTGAGT		
LMCH 34	F-	ATTTGACGGTGTTAAGGTGGT	(GA)11	238-242
	R-	TATGTAGGAAATGACCAGGCTA		

F.PCR products separation and visualization of amplified products

Agarose gel electrophoresis**:** Agarose gel electrophoresis is a molecular technique was used to analyze and separate nucleic acids based on their size. 2µl of amplified PCR products were loaded into each well of 1.5% agarose gel and one well loaded with 0.5 µl of 100bp ladder. Gel electrophoresis was connected to 60V/80V power supply, allowed to run about 2 hr. After the separation, the gel was transferred to EtBr staining tank for 20 min and then washed for 15 min and gel image was taken under the UV light to analyze the PCR products.

G. Polyacrylamide gel electrophoresis (PAGE)

The PCR products were separated on 8% polyacrylamide gel for better separation of the fragments as PAGE gives a higher resolution than agarose gels.

The polyacrylamide gels were prepared with the following recipe as given in the Table 2:

Table 2: Components for preparing Polyacrylamide gel

Component	Quantity
Acrylamide/ Bisacrylamide 29:1 (w/w)	20 ml
5X TBE	15 ml
Distilled water	40 ml
TEMED (N,N.N',N'-Tetramethylenediamine)	28 µl
10 % APS (Ammonium persulphate)	525 µl

The gel was pre-run with 0.5 µl of loading dye for at least 30 min at 200V. 2 µl of PCR product was loaded into the wells and then allowed to run by gel apparatus was connected to a power supply at 200 V for 3 to 3.5 hrs. for migration of DNA fragments to desired resolution.

H. Amplified products scoring

The amplified fragments were scored as 'AA' for the presence of homogenous, 'AB' for the presence of heterogeneous of the alleles from higher to lower molecular weight products and approximate base pair (bp) was determined.

I.SSR DATA ANALYSIS

Based on the PCR results total number of allele, range size of the amplified fragments, effective number of alleles, PIC value, gene diversity, observed (Ho) and expected (He) heterozygosities, and homozygosities calculated for 8 SSRs markers in 34 annona accessions. For population genetic analysis, Popgen version 1.32 software was used.

III.RESULTS AND DISCUSSION

A. PCR temperature optimization

Problems with the amplification of a specific DNA fragment using primers for different species. Nonspecific bands may form after the PCR reaction. Gradient PCR was used to determine the optimal annealing temperature. A previous experiment was given non-specific amplification at the reported annealing temperature of 45°C, 50°C and 55°C for *Annona muricata*. The temperature gradient was set to range from 47 to 57 °C without change the PCR cocktail mixture. Optimum annealing temperature was selected after the gradient PCR products were loaded to 1.5% agarose gel. PCR products band size, bulkiness was considered when selecting the optimum temperature for a particular primer.

Primers LMCH3, LMCH7 and LMCH8 were not gave any bands in agarose gel, primers LMCH1 & LMCH2 were given some non-specific bands. 8 primers (LMCH4, LMCH5, LMCH6, LMCH9, LMCH11, LMCH13, LMCH29& LMCH33) were selected for amplification of *Annona muricata* from out of 16 primers of *A.cherimola* (Table 3).

Table 3: Optimum Annealing Temperature of Selected Primers

Selected primers	Annealing Temperature
LMCH4	55°C
LMCH5	55°C
LMCH6	55°C
LMCH9	55°C
LMCH11	55°C
LMCH13	55°C
LMCH29	57°C
LMCH33	57°C

B.Genetic diversity among species

In this study, the genetic diversity within 34 accessions *Annona muricata* sp. of from various places was evaluated using 8 SSR marker. Therefore, it was possible, to determine whether factors such as geographical origin, source of seeds and improvement status influence the genetic diversity [8]. Amplified product scoring and data analysis were done for each primer based on the band patterns present in the polyacrylamide gel image.

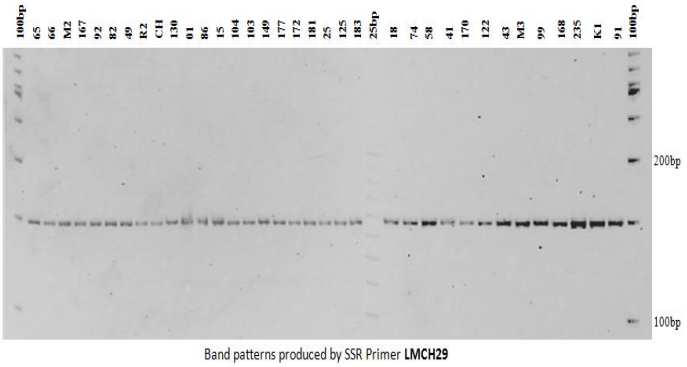

Figure 1: Band patterns produced by SSR primer LMCH29

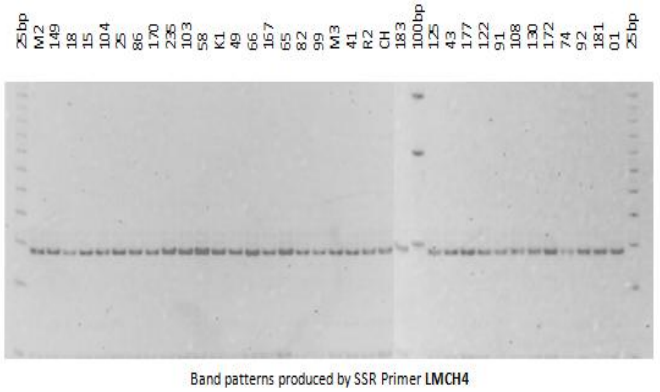

Figure 2: Band patterns produced by SSR primer LMCH4

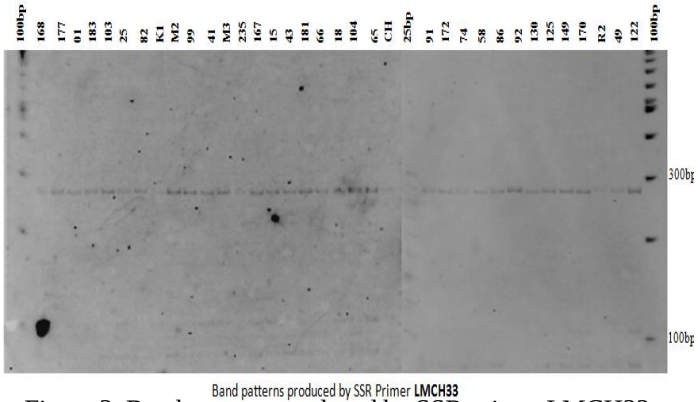

Figure 3: Band patterns produced by SSR primer LMCH33

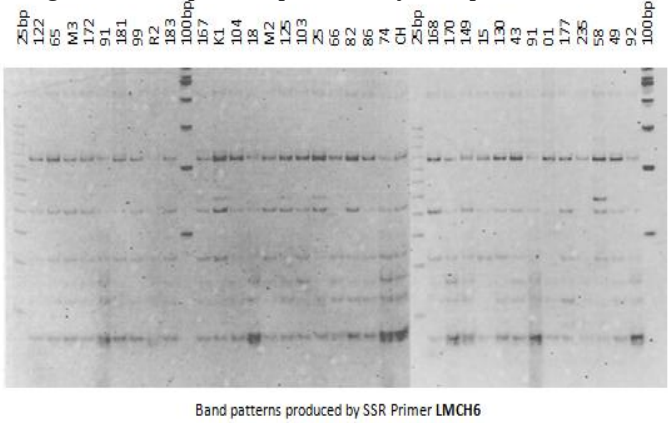

Figure 4: Band patterns produced by SSR primer LMCH6

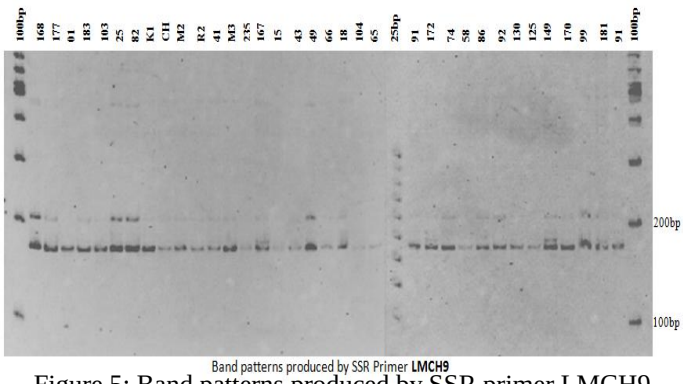

Figure 5: Band patterns produced by SSR primer LMCH9

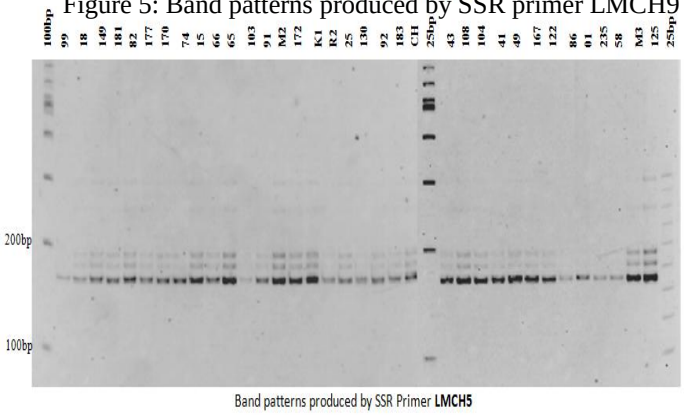

Figure 6: Band patterns produced by SSR primer LMCH5

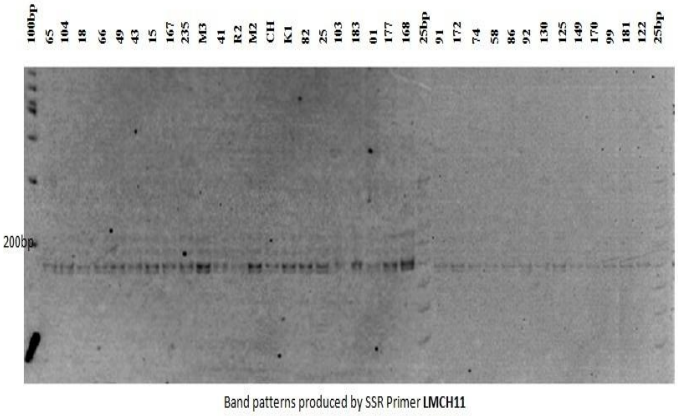

Figure 7: Band patterns produced by SSR primer LMCH11

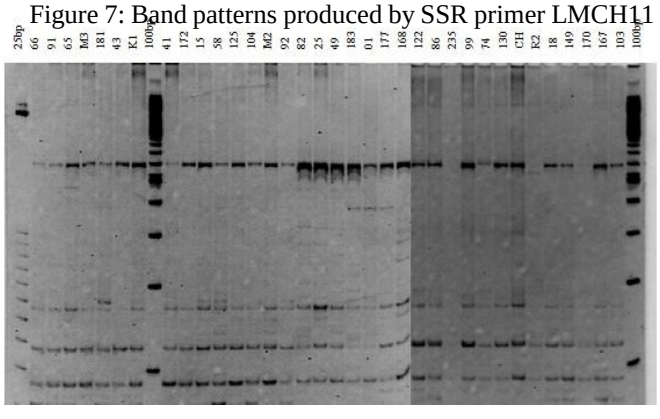

Figure 8: Band patterns produced by SSR primer LMCH13

Table 4: Major allele frequency, Allele No, Gene diversity, Genotype No, Polymorphism Information Content (PIC) and

Marker	Major.Allel.Frqu	Allele No	Gene Diversity	Genotype No	PIC	Product size(bp)
LMCH4	1.0000	1.0000	0.0000	1.0000	0.0000	98
LMCH5	1.0000	1.0000	0.0000	1.0000	0.0000	158
LMCH6	1.0000	1.0000	0.0000	1.0000	0.0000	241
LMCH9	0.9412	2.0000	0.1107	2.0000	0.1046	158-190
LMCH11	1.0000	1.0000	0.0000	1.0000	0.0000	175
LMCH13	1.0000	1.0000	0.0000	1.0000	0.0000	684
LMCH29	1.0000	1.0000	0.0000	1.0000	0.0000	168
LMCH33	1.0000	1.0000	0.0000	1.0000	0.0000	274
Mean	0.9926	1.1250	0.0138	1.1250	0.0131	

Product size of eight SSR markers for 34 Annona accession.

Table 5: Overall Allele Frequency

Allele	LMCH4	LMCH5	LMCH6	LMCH9	LMCH11	LMCH13	LMCH29	LMCH33
A	1.0000	1.0000	1.0000	0.9412	1.0000	1.0000	1.0000	1.0000
B				0.0588				

Table 6: Observes homozygosity (ObsHom), Observes heterozygosity (Obs Het), Expected homozygosity (ExpHom*), Expected heterozygosity (Exp Het*) and Nei's (1973) expected heterozygosity (Nei**).

Locus	ObsHom	Obs Het	ExpHom*	Exp Het*	Nei**
LMCH4	1.0000	0.0000	1.0000	0.0000	0.0000
LMCH5	1.0000	0.0000	1.0000	0.0000	0.0000
LMCH6	1.0000	0.0000	1.0000	0.0000	0.0000
LMCH9	0.8824	0.1176	0.8876	0.1124	0.1107
LMCH11	1.0000	0.0000	1.0000	0.0000	0.0000
LMCH13	1.0000	0.0000	1.0000	0.0000	0.0000
LMCH29	1.0000	0.0000	1.0000	0.0000	0.0000
LMCH33	1.0000	0.0000	1.0000	0.0000	0.0000
Mean	0.9853	0.0147	0.9860	0.0140	0.0138
St.Dev	0.0416	0.0416	0.0397	0.0397	0.0391

Standard Deviation(St.Dev)

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7 SSR markers such as LMCH4, LMCH 5, LMCH6, LMCH11, LMCH13, LMCH29 and LMCH33 showed homozygous alleles. Marker LMCH9 showed polymorphism with heterozygous alleles.

There was a total of 9 genotypes detected across all the loci for 34 accessions of *Annona muricata*. The total number of alleles detected across all the loci was 9. Eight primer pairs amplified same size fragments except primer LMCH9. Locus LMCH9 had 2 alleles and was polymorphic while other loci didn't have any polymorphism. The average number of alleles detected was 1.125 per locus but expected number of alleles 1.0115. The average PIC value for all primers was 0.0131and primer LMCH9 was gave the highest PIC value 0.1046. Major allele's frequency of LMCH9 was 0.9412 but other primers were 1.00 (table 5). The percentage of polymorphic loci was 12.5%. Mean observed heterozygosity and homozygosity for all loci were 0.0147 & 0.9853 respectively, mean expected heterozygosity and homozygosity were 0.0140 & 0.9860 respectively (Table 6). The observed heterozygosity was higher than the expected heterozygosity indicated that mixing of more than two isolated populations. Microsatellite primers developed for a distinct species such as *A.cherimola* can be useful for genetic analysis in related species, but successful transferability depends upon the evolutionary distance between source and target species(4).

In presented results, genetic diversity of *Annona muricata* accessions was limited, these results suggesting a common genetic background similarity for all selected *Annona muricata* accessions. Usually, a relatively low number of SSRs is sufficient to accurately reflect genetic structure and diversity among a high number of accessions (5). So far, there have been no any SSRs markers have been utilized in studies of genetic diversity in *Annona muricata*.

The PIC value of selected SSR markers was relatively low with an average of 0.0131. The average percentage of polymorphic loci was also low (12.5%). The markers transferability varies between the different species of same family because the SSR markers selected for *Annona cherimola* but not modified for *Annona muricata*. The high values of the PIC value and polymorphism results indicated that the primer LMCH9 is well suited for analysis of genetic diversity in *Annona muricata* germplasm. PCR amplified product size of each SSR markers were LMCH6-241bp, LMCH5-158bp, LMCH4-98bp, LMCH9-158-190bp, LMCH33-274bp, LMCH13-684bp, LMCH11-175bp and LMCH29-168bp. Primers LMCH5, LMCH6, LMCH11 AND LMCH13 were produced some scatter bands due to the non-specific PCR amplification of primers. It was suggested that for open pollinated species, the genetic integrity of an accession may be changed in each generation cycle, which can alter the SSR pattern (5).

In the previous experiment, diversity assessment of *Annona muricata* using five ISSR markers(UBC 812*, UBC 841Y, UBC 841 T, UBC 873 and UBC 888)used for 29 accessions of same selected *Annona muricata* plants collected from Agriculture Research Station (ARS), Girandurukotte was gave different results compare to SSR markers. Each ISSR marker amplified with seven to thirteen bands with an average number of 9.4 bands per marker and81% of the generated bands were found polymorphic. Mean gene diversity of selected *A.muricata* germplasm was 0.2233 and the highest diversity was shown by the fourth locus of UBC 812* ISSR marker (2). Accession number 43 and 149 gave the highest genetic identity of ninety eight percent (2).

These studies demonstrate that DNA variation exists among *Annona muricata* genotypes and although identified polymorphism but level of genetic variation is very limited. Accessions 168, 25, 82 & 49 were given heterozygous alleles band for primer LMCH9.These variations in the annona accessions might mutation caused by an error in the replication of DNA, translocations, duplication- single nucleotides removals, substitutions, or insertions.

IV.CONCLUSIONS

As a result of genetic diversity study in *Annona muricata* species was revealed a relatively limited genetic diversity within selected *Annona muricata* accessions with respect to *Annona cherimola* markers. Annealing temperature of 6 selected primers (LMCH4, LMCH5, LMCH6, LMCH9, LMCH11, LMCH13) were 55°C and other 2 selected primers (LMCH29, LMCH33) were 57°C. 7 SSR markers (LMCH4, LMCH5, LMCH6, LMCH11, LMCH13, LMCH29 & LMCH33) have low level of polymorphism for identify the genetic diversity of *Annona muricata* species within that primer LMCH9 has high level of polymorphism. Accessions 168, 25, 82 & 49 were gave heterozygous alleles band for primer LMCH9.

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