MOLECULAR ANALYSIS OF MANGO (*Mangifera indica* L.) Cv. ALPHONSO FROM DIFFERENT LOCATIONS OF SOUTH KONKAN

By

Mr. PATIL VINAYAK BABANRAO

B. Sc. (Ag. Biotechnology)

PLANT BIOTECHNOLOGY CENTRE,

FACULTY OF AGRICULTURE,

Dr. BALASAHEB SAWANT KONKAN KRISHI VIDYAPEETH, DAPOLI - 415 712,

DIST. RATNAGIRI (M. S.)

MAY, 2019

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A thesis submitted to the

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DAPOLI
(AGRICULTURAL UNIVERSITY)
DIST. RATNAGIRI (MAHARASHTRA STATE), INDIA

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE (AGRICULTURE)

In

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Approved by the Advisory Committee:
Chairman and Research Guide:

(S. V. Sawardekar)
In-charge
Plant Biotechnology Centre,
College of Agriculture, Dapoli.

Members:

(R. S. Deshpande)
Associate Professor (CAS),
Plant Biotechnology Centre,
College of Agriculture, Dapoli.

(R. G. Khandekar)
Professor,
Department of Horticulture,
College of Agriculture, Dapoli.

(M. G. Palshetkar)
Assistant Professor,
Department of Agril. Botany,
College of Agriculture, Dapoli

Dr. S. V. SAWARDEKAR
In-charge
Plant Biotechnology Centre,
College of Agriculture, Dapoli.
Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth,
Dapoli – 415 712, Dist. Ratnagiri (M. S.)

CERTIFICATE

This is to certify that the thesis entitled, **"MOLECULAR ANALYSIS OF MANGO (*Mangifera indica* L.) Cv. ALPHONSO FROM DIFFERENT LOCATIONS OF SOUTH KONKAN"** submitted to the Plant Biotechnology Centre, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist. Ratnagiri, Maharashtra State, in the partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (AGRICULTURE) in AGRICULTURE BIOTECHNOLOGY,** embodies the results of a piece of bona-fide research carried out by **Mr. PATIL VINAYAK BABANRAO** (Regd. No. 0029) under my guidance and supervision and that no part of this thesis has been submitted for any other degree or diploma or published in other form. All the assistance and help received during the course of investigation and the sources of literature have been duly acknowledged by him.

Place: Dapoli

Date : 31st May, 2019

(S. V. Sawardekar)
Chairman,
Advisory Committee
and
Research Guide

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CHAPTER I

INTRODUCTION

Mango (*Mangifera indica* L., Family: Anacardiaceae, Chromosome number: 2n = 40) is one of the oldest and most important tropical fruits worldwide. It is rightly known as 'King of fruits' owing to its nutritional richness, unique taste, and pleasant aroma. It is also considered as 'National fruit of India'. It originated in the South East Asian or Indo-Burma Region and has been cultivated for 4000 years in India (Mukherjee, 1953; Kostermans and Bompard, 1993). India is the largest producer of mango in the world followed by China, Thailand, Indonesia, and Mexico (Anonymous, 2018a).

In India the area and production of mango has been almost continuously increases over the years. The area under mango cultivation was 1077.6 thousand ha during 1991-92 which reaches up to 2516 thousand ha in 2013-14 and recorded 2262.8 thousand ha in 2016-17. However the production has been fluctuating drastically. A total 109.99% increase in area under mango cultivation has been recorded from 1991-92 to 2014-15 while, 125.88% increase in production was recorded during the same period. The productivity of 8.1 MT/ha was recorded during 1991-12 whereas, it was declined up to 5.5 MT/ha in 2014-15 and reached up to maximum productivity of 8.7MT/ha in 2016-17. There was overall increase of 7.41% in productivity of mango from 1991-92 to 2014-15.

In Maharashtra within 2014-15 mango area is 157.77 ha, production of mango is 758.84 MT and productivity is 4.8 MT/ha. In year 2015-16 mango area is 162.08 ha, production of mango is 463.17 MT and productivity is 2.9 MT/ha. Recent year 2016-17 mango area is 157.07 ha, production of mango is 514.87 MT and productivity is 3.3 MT/ha.

Table 1: Area, Production and Productivity of Mango in India.

Year	Mango Area (in ha)	Production (in MT)	Productivity (in MT/ha)
2010-11	2297	15188	6.6
2011-12	2378.1	16196.4	6.8
2012-13	2500	18002.4	7.2
2013-14	2516	18431.3	7.3
2014-15	2163.5	18527	8.5
2015-16	2208.6	18642.5	8.4
2016-17	2262.8	19686.9	8.7

(Anonymous, 2018b)

Nearly 1000 varieties of mango are grown in India. The important among them are Alphonso, Beneshan, Badami, Chausa, Dasheri, Bombay Green, Pairi, Mulgoa, Sindoori, Raspuri, Gulaab Khaas, Amrapali, Kishenbhog. Among all these varieties Alphonso is the choicest variety.

Alphonso is one of the most popular mango variety of India. In Konkan, about 90 per cent area of mango is occupied by single cultivar "Alphonso", which is locally called as 'Hapus'. It thrives and yields best under warm and humid climate of Konkan region (Burondkar, 2005). The variety was named after Afonso de Albuquerque a Portuguese general and military expert who helped to establish Portuguese colonies in India. The Portuguese introduced grafting on mango trees to produce extraordinary varieties like Alphonso. The fruit was then introduced to the Konkan region in Maharashtra, Goa, Gujarat and some parts of southern states of Tamil Nadu, Karnataka and Kerala. This variety has major export share to the tune of over 35 per cent (Burondkar, 2005). Alphonso is recognized nationally and internationally for its attractive fruit shape, color, flavor, taste and aroma besides excellent keeping quality after

ripening. It is suitable for table purpose and export. This variety not only preferred in Indian metropolitan markets but also in export especially to the countries like UAE, Soudi Arabia, UK, USA etc.

Alphonso fruits are medium size with excellent sugar acid blend and good keeping quality. Its flavor is retained even after processing and hence preferred in processing industry (Cheema and Dani, 1934). Alphonso mangoes have a rich, creamy, tender texture and are low in fiber content, with delicate, creamy pulp. These characteristics make Alphonso one of the most in-demand cultivars. The skin of a fully ripe Alphonso mango turns bright golden yellow with a tinge of red which spreads across the top of the fruit. The flesh of the fruit is golden saffron color.

The molecular analysis is an important for mango improvement programs and management of genetic resources. In mango growing regions, breeding attempts are always in progress for creating better cultivars. The main objectives of mango breeding aimed to improve both plant and fruit characteristics such as dwarf trees, profuse and regular bearing, good fruit size and edible quality, less fibers, attractive peel and pulp color, diseases resistance and long storage life (Usman *et al.*, 2001). Precise information is needed for carrying out efficient breeding programs. In order to analyze molecular makeup in Alphonso, PCR-based DNA markers are among the best tool. Unlike the agronomic and morphological characteristics, the molecular markers are not subjected to the environmental effect. Recently, the DNA markers generated by PCR methods have been used in mango characterization (Singh *et al.*, 2009; Bhargava and Khorwa, 2011; Begum *et al.*, 2014). Among these all PCR-based DNA markers, Inter Simple Sequence Repeat (ISSR) has huge advantages.

Inter simple Sequence Repeats or ISSR have been proven useful for detecting genetic polymorphisms among accessions by generating a large number of markers that target multiple microsatellite loci distributed across the genome. ISSR are often used because of their capability of reproducibility, with no gene sequence information, and prior genetic studies are required for the analysis. ISSR markers have been reported to undertake the assessment of genetic diversity of mango genotypes (Pandit *et al.*, 2007). ISSRs offer greater probability than any other PCR marker system in the repeat regions of the genome, which are the most potent regions for producing cultivar-specific markers. This is also the attribute of ISSRs which renders them useful as a supplementary system to any of the random and dominant marker system. Automated PCR base makes ISSRs the markers of choice for screening genotypes (Pandit *et al.*, 2007).

In Konkan, Devgad Alphonso (Hapus) has signature characteristics like the aroma, no fiber and sweet taste. These characteristics are ascribed to geographical conditions of Devgad and not observed in varieties grown elsewhere. As we move from South Konkan to North Konkan, Alphonso differ in taste. Very few research has been done on phenotypic characters of Alphonso of South Konkan and other region of Konkan. But the genotypic information of Alphonso is lacking.

On account of this, experiment was conducted with the following objectives:

1. To establish Alphonso mango genotype profiles through ISSR markers.
2. To analyze genetic variability of Alphonso mango through ISSR marker.

3. CHAPTER II

4. REVIEW OF LITERATURE

5. A brief review of literature related to the present study entitled, "Molecular analysis of Mango (*Mangifera indica* L.) Cv. Alphonso from different locations of South Konkan" is discussed in this chapter under different headings.
6. Traditionally, evaluation and conservation of bio-diversity/genetic variability is based on comparative anatomy, morphology, embryology, physiology, *etc.*, which provide informative data but of low genetic resolution. The research work on the diversity of fruit in general and mango in particular has been reviewed in this chapter. The Alphonso variety of mango cultivated in the konkan region varies in taste at different location. To ascertain whether this variation is due to the genetic diversity or it is an environmental impact, this study has been undertaken. Molecular analysis of a species is a prerequisite for future sustainable breeding efforts. Molecular marker based genotyping involves the development of marker profile unique to an individual. Molecular markers provide an important technology for evaluating levels and patterns of genetic diversity and have been utilized in a variety of plant species. The various DNA marker methods currently available that can be used to examine genetic diversity at the molecular level. Among the different molecular markers, the ISSR technique is an effective, rapid, simple, reproducible and inexpensive way to assess genetic variability or to identify closely related cultivars in many species (Moreno *et al.*, 1998).
7.
8.

9. **2.1 Standardization of DNA isolation protocol**

10. Doyle and Doyle (1990) gave a rapid DNA isolation procedure for small quantities of fresh leaf tissue.

11. Edwards *et al.*, (1991) gave a simple and rapid method for the preparation of plant genomic DNA for PCR analysis. The polymerase chain reaction (PCR) has revolutionized the rapid analysis of mammalian genomic DNA. However, PCR is less useful in the analysis of plant DNA due to the difficulties in extracting nucleic acids from limited amounts of plant tissue. They have developed a method for the rapid extraction of small amounts of plant genomic DNA suitable for PCR analysis. The method is applicable to a variety of plant species and has the added advantage of not requiring any phenol or chloroform extraction. Thus it is possible to complete an extraction within 15 minutes without handling any hazardous organic solvents.

12. John (1992) described the modified protocol of DNA extraction including higher buffering capacity, alkaline pH, and most importantly poly vinyl pyrrolidone, which through hydrogen bonding, complexes with polyphenolics, effectively removing them from the homogenate. The homogenate is then subjected to ultracentrifugation to isolate RNA or DNA. The isolated RNA is suitable for cDNA cloning, *In-Vitro* translation or polymerase chain reaction. Plant tissues (leaf, fiber, root, stem, ovules or flowers) were quick frozen in liquid nitrogen. The frozen tissue was powdered in a mortar in liquid nitrogen and then homogenized in a buffer for 1.5 minutes using a polytron at full speed. The homogenization buffer was added at a ratio of 1:2 of tissue (weight) to buffer (volume). In some instances it was found that increasing the ratio to 1:4 enhances the yield.

Homogenization buffer was; 5 M guanidine isothiocyanate, 0.2 M Tris-acetate (pH 8.5) 0.7% 3-mercaptoethanol, 1% polyvinyl pyrrolidone (soluble PVP, MW 40 Kd), and 0.62% sodium lauryl sarcosine). Both 3-mercaptoethanol and PVP were added to the buffer just before homogenization. PVP should be prepared fresh as a 20% stock each time. The homogenate was filtered through Mira cloth and layered over a 1.5 ml pad of 5.7 M cesium chloride. The homogenate was then centrifuged for 18 hours at 36,000 rpm in a SW 50.1 rotor at 20°C and then further purified by phenol: chloroform extractions and precipitations in the presence of ammonium acetate. Omitting the PVP in the homogenization buffer yielded RNA that could not be translated *In Vitro* systems. In both instances the homogenization buffer was modified to contain 1 % PVP.

13. Stewart and Laura (1993) gave a rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. Many DNA isolation techniques widely employed by plant molecular biologists use a CTAB (Cetyl Trimethyl Ammonium Bromide) extraction buffer coupled with reusable tissue homogenization systems such as a mortar and pestle. These procedures, though simple, typically use large amounts of buffer (10 ml), utilize nondisposable homogenizers and require ethanol washes. The risk of cross contamination associated with reusing homogenizers and vessels is unacceptable if the DNA isolated will be amplified in PCR or RAPD (random amplified polymorphic DNA), experiments. Recent DNA extractions methods developed to avoid potential contamination disrupt cells by biochemical means, leaf squashes or sodium dodecyl sulfate mini preps. However, the

biochemical lysis method and the leaf squash method are complicated and/or do not yield sufficient DNA for many replicate reactions. The SDS procedure is similar to the protocol described here, but the CTAB buffer should be more amenable to plant material containing polysaccharides.

14. Kim *et al.,* (1997) reported a simple and rapid method for isolation of high quality genomic DNA from fruit trees and conifers using PVP. Because DNA degradation is mediated by secondary plant products such as phenolic terpenoids, the isolation of high quality DNA from plants containing a high content of polyphenolics has been a difficult problem. They demonstrate an easy extraction process by modifying several existing ones. Using this process they have found it possible to isolate DNAs from four fruit trees, grape (*Vitis* spp.), apple (*Malus* spp.), pear (*Pyrus* spp.) and persimmon (*Diospyros* spp.) and four species of conifer, *Pinus densiflora*, *Pinus koraiensis*, *Taxus cuspidata* and *Juniperus chinensis* within a few hours. Compared with the existing method, they have isolated high quality intact DNAs (260/280 = 1.8–2.0) routinely yielding 250–500 ng/µl (total 7.5–15 mg DNA from four to five tissue discs.

15. Khanuja *et al.*, (1999) isolated DNA by rapid method from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. The presence of certain metabolites have been observed to interfere with DNA isolation procedures and downstream reactions such as DNA restriction, amplification and cloning. The chemotypic heterogeneity among species may not permit optimal DNA yields with a single protocol, and thus, even closely related species may require different isolation protocols. Here they describe the

essential steps of a rapid DNA isolation protocol that can be used for diverse medicinal and aromatic plants, which produce essential oils and secondary metabolites such as alkaloids, flavanoids, phenols, gummy polysaccharides, terpenes and quinones. The procedure is applicable to dry as well as fresh plant tissues. This protocol, in their experiments, permitted isolation of DNA from tissues of diverse plant species and produced fairly good yields.

16. Dilworth and Frey (2000) described a rapid and reliable method for high throughput extraction of DNA from plant material using glass beads in a flat-bottomed microtitre plate. This procedure was quick, inexpensive, and allows up to 96 samples to be processed in parallel. PCR products produced by the recovered DNA are consistently equivalent to those produced through traditional extraction methods.

17. Manimekalai *et al.*, (2004) optimized DNA amplification fingerprinting parameters in coconut (*Cocos nucifera* L.). DNA Amplification Fingerprinting (DAF) is a simple and powerful technique in genome analysis. It is being employed in various applications in a number of crops. For obtaining reproducible result from DAF technique optimization of PCR parameters was done. 20 ng (2ng/µl) template DNA, 25 µM primer, 250 µM each of dNTP's, 2 U (0.2 U/µl) Taq polymerase, 25mM $MgCl_2$ and annealing temperature of 55°C with standard silver staining protocol gave good amplification with reproducibility.

18. Ramirez *et al.*, (2004) isolated genomic DNAs from the tropical fruit trees mango, guava, coconut, and avocado for PCR-based DNA marker application. With tropical fruit trees, the isolation of genomic DNA with sufficient quality for the

application of PCR-based DNA marker technology very often has severe problems due to the presence of inhibitors such as polysaccharides, which inhibit enzymatic DNA processing or polyphenols as inhibitors of PCR reactions. Here, different protocols for DNA extraction and purification were tested with the four tropical fruit trees guava (*Psidium guajava* L.), avocado (*Persea americana* Mill.), mango (*Mangifera indica* L.) and coconut (*Cocos nucifera* L.). The well-established CTAB protocol of Doyle and Doyle yielded excellent DNA templates for PCR amplification with mango and coconut, but not so with guava and avocado. Modification of the CTAB method with respect to CTAB buffer composition and in combination with reversible adsorption to Nucleo Spin columns alleviated the problems encountered with the genomic DNA of both species.

19. Angeles *et al.*, (2005) reported the extraction of genomic DNA from the lipid, polysaccharide, and polyphenol-rich coconut (*Cocos nucifera* L.). Genomic DNA extracted from the young leaves of the first emergent frond provided enzyme digestible, good-quality DNA. The modification involved the use of a higher salt concentration (2 M instead of 0.5 M) in the extraction buffer and the use of polyvinylpolypyrrolidone (PVP). Moreover, this modified protocol did not involve the use of organic solvents.

20. Preetha and Subramanian (2007) studied the isolation and quantification of DNA. They reported that the DNA extraction process involves separation of DNA from naturally occurring plant cell constituents such as polysaccharides and polyphenolic compounds.

21. Kit and Chandran (2010) studied a simple, rapid and efficient method of isolating DNA from Chokanan mango (Mangifera indica L.). Total DNA of Chokanan mango (Mangifera indica L.) was extracted from the leaf for the construction of total genomic library. However, the quality of the extracted DNA was often compromised by the presence of secondary metabolites, thus interfering with the analytical applications. Improvement on the quality of the extracted DNA was achieved through the optimization of leaf harvesting stag and modification on the cetyl trimethyl ammonium bromide (CTAB) DNA extraction procedure. Fully expanded, soften and purplish leaf was proved to yield good DNA quality while the addition of polyvinylpyrrolidone (PVP) and β-mercaptoethanol was effective in the removal of secondary metabolites, particularly polyphenolic compounds. The incorporation of high salt washing step was also efficient in removing polysaccharides. This simple, inexpensive and yet reliable method was proved to be successful in yielding sufficient quality and quantity of DNA for the construction of genomic library.

22. Majumder *et al.*, (2011) developed the simple and efficient method for genomic DNA extraction from woody fruit crops containing high polysaccharide levels. In this study, three kinds of plant DNA extraction protocols were studied and the target was to establish the water-saturated ether (WSE) with 1.25 M NaCl method as the most efficient protocol for removing the highly concentrated polysaccharides from genomic DNA of woody fruit crops. This method involves the modified CTAB or SDS procedure employing a purification step to remove polysaccharides using the WSE method. Precipitation with an

equal volume of isopropanol caused a DNA pellet to form. After being washed with 70% ethyl alcohol, the pellet became easily dissolved in TE buffer. Using these three methods, DNA was extracted from samples of 60 mango genotypes, including young, mature, old, frosted old and withered old leaves. Compared with the three studied DNA extraction protocols of mango, it was found that the WSE method with NaCl had the highest value of average percentage (85.44%) in DNA content of the mango genotypes. The average yield of DNA ranged from 5.05 µg/µL to11.28 µg/µL. DNA was suitable for PCR and RAPD analyses and long-term storage for further use.

23. Sahu *et al.*, (2012) studied DNA extraction protocol for plants with high levels of secondary metabolites and polysaccharides without using liquid nitrogen and phenol. Mangroves and salt marsh species are known to synthesize a wide spectrum of polysaccharides and polyphenols including flavonoids and other secondary metabolites which interfere with the extraction of pure genomic DNA. This study describes a rapid and reliable cetyl trimethyl ammonium bromide (CTAB) protocol suited specifically for extracting DNA from plants which are rich in polysaccharides and secondary metabolites, and the protocol also excludes the use of expensive liquid nitrogen and toxic phenols. Purity of extracted DNA was excellent as evident by A260/A280 ratio ranging from 1.78 to 1.84 and A260/A230 ratio was >2, which also suggested that the preparations were sufficiently free of proteins and polyphenolics/polysaccharide compounds. DNA concentration ranged from 8.8 to 9.9 µg μL^{-1}.
24. Huang *et al.*, (2013) reported an efficient DNA isolation method for tropical plants. Due to interfering components such

as polysaccharides, polyphenols, etc., DNA isolation from tropical plants had been challenging. They developed a safe, universal and efficient DNA extraction method, which yielded high-quality DNA from 10 tropical plants including cassava, rubber tree, banana, etc. In the extraction buffer, 2 M NaCl was used to provide a high ionic strength reaction environment, ethylene diamine tetra acetic acid (EDTA), lauryl sarcosine (LSS) and cetyl trimethyl ammonium bromide (CTAB) could inhibit DNase activity effectively, polyvinylpolypyrrolidone (PVPP) produced a deoxidized reaction environment, and borax enhanced the precipitation of interfering compounds. Ordinary reagents like β-mercaptoethanol, chloroform and phenol were unnecessary in this protocol, which made it safe and friendly to use. PCR and Eco RI enzyme restriction digestion results show that the obtained DNA is good enough for downstream analysis. In conclusion, this protocol is expected to be a preferable DNA extraction protocol for tropical plants.

25. Azmat *et al.*, (2014) described a reliable and modified protocol based on the cetyl trimethyl ammonium bromide (CTAB) method for DNA extraction from mature mango leaves. High concentrations of inert salt were used to remove polysaccharides; polyvinyl pyrrolidone (PVP) and β-mercaptoethanol were employed to manage phenolic compounds. Extended chloroform-isoamyl alcohol treatment followed by RNase treatment yielded 950–1050 µg of good quality DNA, free of protein and RNA. The problems of DNA degradation, contamination, and low yield due to irreversible binding of phenolic compounds and precipitation of polysaccharides with DNA were avoided by this method. The

DNA isolated by the modified method showed good PCR amplification using simple sequence repeat (SSR) primers. This modified protocol can also be used to extract DNA from other woody plants having similar problems.

26. Sharma *et al.*, (2014) compared six different methods of DNA isolation namely, CTAB, CTAB with PVP, Qiagene DNA extraction Mini and Maxi kits, CTAB with addition of PVP and purification using PCI followed by Qiagen Genomic-tip 500/G were for recovery of quality DNA from mango leaves. The higher yield (1375.0 ng/ µl) of good quality (A260/280 and A260/230 1.80 & 1.90, respectively) DNA was obtained with modified CTAB method with addition of PVP (MW, 40,000) followed by purification using phenol: chloroform: isoamyl: alcohol 25:24:1 and Qiagen Genomic-tip 500/G as compared to standard CTAB method (1096.50 ng/ µl; A260/280 and A260/230 1.40 and & 1.10, respectively). The DNA obtained using modified CTAB method was found suitable for PCR, PacBio, ddRAD sequencing and long-term storage.

27. Uddin *et al.*, (2014) employed an additional step to remove polysaccharides, polyphenols and secondary metabolites from genomic DNA extracted from young or mature leaf tissue; then a modified traditional cetyl trimethyl ammonium bromide (CTAB) method was applied. The use of 0.4 M glucose improved DNA quality and avoided contamination and browning by polyphenols, relative to the traditional CTAB method. This is an easy and efficient method for genomic DNA extraction from both young and mature leaves of mango. The isolated DNA was free of polysaccharides, polyphenols, RNA and other major contaminants, as judged by its clear color, its viscosity,

A260/A280 ratio and suitability for PCR-based reactions. This modified protocol was also used to extract high quality genomic DNA from other woody perennials, including walnut, guava, lychee, pear, grape and sugarcane.

28. Sinha and Kumar (2017) evaluated NaOH-Tris DNA extraction method for PCR analysis because this is very simple, time saving and safe without the need to use expensive or rare materials and laboratory apparatus. This method only requires a small amount of leaf tissue, NaOH, Tris, micro tube and plastic pestle. The amplified PCR products showed clear, sharp and uniform bands gave similar results as compared with the modified CTAB method. The DNA obtained is crude contains impurities like protein, RNA but these impurities did not affect PCR amplification. This DNA extraction method is evaluated for brinjal (*Solanum melongena* L.), chilli (*Capsicum annuum* L.), rice (*Oryza sativa* L.) and tomato (*Solanum lycopersicum* L.) crop. Many other crop plants could also be amplified using the same DNA extraction method for molecular analysis of large samples. Thus, the use of NaOH-Tris method will allow researchers to obtain DNA from plant quickly for use in molecular studies.

29.

30.

31. 2.2 Standardization of Polymerase chain reaction (PCR) protocol

32. Padmalatha and Prasad (2006) optimized the DNA isolation and PCR conditions for RAPD analysis of selected medicinal and aromatic plants of conservation concern from Peninsular India containing high levels of polysaccharides,

polyphenols and secondary metabolites. The method involves a modified CTAB extraction employing polyvinyl pyrrolidone while grinding, successive long-term Chloroform: lsoamyl alcohol extractions, an overnight RNase treatment with all steps carried out at room temperature. The yield of DNA ranged from 1-2 µg/µl per gram of the leaf tissue and the purity (ratio) was between 1.6-1.7 indicating minimal levels of contaminating metabolites. RAPD protocol was optimized based on the use of higher concentration of $MgCl_2$ (3 mM), lower concentrations of primer (0.5 µM) and *Taq* polymerase (0.2 units), 50 ng of template DNA and an annealing temperature of 37°C, resulted optimal amplification.

33. Azhar *et al.*, (2013) evaluated the optimum condition for each PCR reactions at 1x PCR Buffer, 1.5 mM $MgCl_2$, 0.8 mM dNTP mixed, 0.8 µM primers, 60 ng/µl DNA template and 0.5 U *Taq* polymerase. The total amounts of each reaction was set at 25 µl. These condition was used further in optimizing annealing temperature for the evaluation of the 100 ISSR primers.

34. Kumari *et al.*, (2015) optimized ISSR protocol for Gladiolus (Gladiolus x hybridus Hort.), the queen of the bulbous ornamentals for template DNA, dNTP, primer, and Taq polymerase concentration and annealing temperature. Template DNA of 80 ng, 1mM of dNTP, 0.33 U of *Taq* DNA polymerase, 1 µM primer gave highly reproducible PCR products. Reproducible amplifiable products were observed in all PCR reactions.

35. Mohamad *et al.*, (2017) examined 100 ISSR primers as well as, 56 ISSR primers was productively amplified. Optimum components for PCR reactions were 5.0 µl of 5X PCR Buffer, 1.5 µl of 25mM MgCl2, 1 µl of 10 mM dNTP, 1 µl of 10 mM ISSR

primers, 2 µl Template DNA, and 0.1 µl of 5 units/ml *Taq* polymerase. Based on this study, has brought out some information on the relationship between these ISSR primers will be applied further for molecular profiling as well as response evaluation in rice varieties.

36. **2.3 Molecular analysis of mango by RAPD, ISSR and SSR markers**

37. Schnell and Knight (1993) analyzed 9 mangifera species for molecular characterization by using DNA based fingerprinting technique for first time to determine the phylogenetic relationship. The phenogram generated did not support the classification of Kostermans and Bompard (1993) in which they divided Mangifera into two subgenera viz. Mangifera containing four selection and *Limus* containing two selections. Kostermans and Bompard (1993) placed the species *M. casturi, M. indica, M. laurina, M. quadrifida* and *M. torquenda* in the subgenus *mangifera,* selection *mangifera* within this section, *M. quadrifida* and *M. torquenda* were considered to be closely related to each other and *M. casturi, M. indica* and *M. laurina* were considered to be taxonomically closer. The other species belong to the subgenus *Limus*, with *M. decandra* in section Deciduae and *M. foetida*, *M. odorata* and *M. pajang* in section perennis.

38. Bally *et al.*, (1996) investigated the genetic diversity of Kensington mangoes (*Mangifera indica* L.) by using random amplified polymorphic DNA (RAPD) analysis. DNA was extracted from leaves of 27 'Kensington Pride', 2 'R2E2' and 1 seedling. RAPD analysis with 10 oligonucleotide primers allowed the scoring of 107 markers. The R2E2 trees (20% dissimilarity) and

the seedling (10% dissimilarity) were distinct from the Kensington Pride. However, there was very little evidence of significant genetic variation within Kensington Pride selections. Fifteen of the selections were identical in all 107 markers. Only 2 selections, WEAN2 and ML2N1, differed by more than 5%. Many of the differences found in Kensington mango orchards may be due to environmental factors not genetic variations.

39. Jayasankar *et al.*, (1998) studied the use of RAPD as a marker for variation in embryogenic mango cultures following *In-Vitro* selection with *Colletotrichum gloeosporioides* culture filtrate of two mango cultivars "Hindi" and "Carabao". *In-Vitro* selection caused changes in RAPD markers in the selected embryonic cultures with respect to unchallenged controlled cultures and stock plants. The differences involved both the absence and presence of additional RAPD markers in resistant lines although, the former was most commonly observed.

40. Kashkush *et al.*, (2001) used Amplified Fragment Length Polymorphism (AFLP) for identification of mango (*Mangifera indica L.*) cultivars, for studying the genetic relationship among 16 mango cultivars and seven mango rootstocks and for the construction of a genetic linkage map. Six AFLP primer combinations produced 204 clear bands and on the average 34 bands for each combination. The average band-sharing between cultivars and rootstocks was 83% and 80%, respectively. The average band-sharing for mango is 81%. The probability of obtaining a similar pattern for two different mango cultivars and rootstocks is 6×10^{-3} and 2×10^{-3}, respectively. A preliminary genetic linkage map of the mango genome was constructed, based on the progeny of a cross between 'Keitt' and 'Tommy-

Atkins'. This linkage map consists of 13 linkage groups and covers 161.5 cM defined by 34 AFLP markers.

41. Karihaloo *et al.*, (2003) demonstrated that, differences among regions were significant; northern and eastern region formed one zone and western and southern regions formed another zone of mango diversity in India. By taking 29 Indian mango cultivar comprising popular land races, advance cultivar and random amplified polymorphic DNA analysis. PCR amplification with 24 primers generated 314 bands 91.4% of which were polymorphic. Jaccard's similarity co-efficient between pair of cultivars ranged between 0.318 and 0.75 with a mean of 0.565. A UPGMA dendrogram showed the majority of cultivar from northern and eastern regions of India clustering together and separate from southern and western cultivar. Analysis of molecular variance revealed that 94.7% genetic diversity in mango existed within region.

42. Pandit *et al.*, (2007) analyzed genetic diversity of 70 mango cultivars and distant out-group with 33 polymorphic ISSR markers. Clustering was done using Dice and Jaccard's coefficients with bootstrapping. Multivariate analysis was conducted using the Euclidean distance. Among the total 420 bands, 408 were polymorphic. Probability that any ISSR fragment is shared by two different cultivars was calculated to be 2.54×10^{-1}. Non- Indian mango cultivars were found genetically diverged from Indian mango gene pool. Twelve different cultivar-specific bands were detected for six cultivars, which approved the effectiveness of ISSR markers in mango genetic diversity analysis.

43. Singh *et al.*, (2007) profiled twelve Indian mango cultivars by using anchored-ISSR primers. Out of total 161 bands amplified, 113 (70.2%) were polymorphic, the polymorphism ranging from 50% to 94.1% depending upon the primer. One primer [5´ HVH(CA)7T 3´] with highest genotype index could uniquely identify each of the cultivars studied. Fingerprints based on polymorphic markers amplified by the 10 primers had a 6.75 x 10-12 probability of identical match by chance, indicating a high degree of uniqueness in the anchored-ISSR based fingerprinting. UPGMA dendrogram based on Jaccard's similarity showed Himayath as the most diverse of the 12 cultivars and the rest of the cultivars clustered into two groups at 0.69 similarity coefficient.

44. Manchekar (2008) examined nine Alphonso mango clones using Random Amplified Polymorphic DNA (RAPD) markers with decamer primers of arbitrary sequence. Seven of the eight primers screened were informative and 36 amplified DNA bands were selected as RAPD markers. Clusters analyzed based on seven RAPD markers produced a dendrogram of the genetic relatedness among the nine Alphonso clones. The clones *viz.,* DEV-I, RTN-I, VEN-I, DPL-I, DWR-I, DWR-II and DWR-III were most similar and formed into one cluster, whereas, BGM-I and BGM-II formed another.

45. Mansour *et al.*, (2008) conducted analytical studies on mango fruit crop in Egypt. Utilization of molecular marker analysis provided new insights to breeders for molecular assisted selection (MAS). Depending on the marker system used, the genetic similarity analyses varied dramatically. Genomic variation within twelve mango cultivars, widely used in

fresh market mango production in Egypt, were investigated using two different molecular marker systems; RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeat). A new strategy was used to increase RAPD potential in genetic diversity by using three different primer combinations per reaction. Different dendrograms constructed for the RAPD and ISSR results individually and collectively revealed that similarity and clustering is very dependent on the marker system used.

46. Díaz-Matallan *et al.*, (2009) studied the genetic diversity of six populations of mango Hilacha by RAPDs markers, as a fundamental base for breeding programs, conservation and selection of promissory materials for the fruit industry at the national level. From 60 primers evaluated in the populations, five primers were selected and were launched in the six populations. Polymorphic bands of RAPDs were transformed into binary matrices, which were then processed with NTSYS-PC, POPGENE and TFPGA softwares. The overall genetic diversity, HT = 0.468 + 0.0016, is very similar to the average subpopulation genetic diversity, HS = 0.4431 + 0.0024, which revealed a small genetic differentiation among the mango Hilacha populations studied (GST = 0.0532). This means that each population contained in average 95 % of the total genetic diversity found in the global population analyzed. Considerable gene flow between populations (Nm = 9) was found.

47. Singh *et al.*, (2009) investigated the genetic diversity of five commercially important mango cultivars of India, comprising three landraces ('Banganapalli', 'Dashehri', and 'Langra') and two recently-bred cultivars ('Amrapali' and 'Mallika') by using

morphological and molecular Inter Simple Sequence Repeat (ISSR) markers. Morphological analysis based on 17 fruit characters detected prominent variation in the landraces 'Banganapalli', 'Langra', and 'Dashehri' and some variation in the cultivar 'Mallika'. Using ten ISSR repeat primers, intracultivar variation was detected among replicates of 'Banganapalli', 'Langra', and 'Mallika', while replicates of 'Amrapali' and 'Dashehri' showed no variation. The 20 replicates of 'Mallika' showed four distinct fingerprints differing from each other in the presence or absence of five bands all produced by a single primer, 5'HVH(TCC)53'. In 'Langra', all but one replicate had identical molecular fingerprints while in the 15 'Banganapalli', four different fingerprints were identified. Some replicates showed both morphological and molecular differences from the other replicates of the respective cultivars suggesting a molecular basis of the observed morphological variation. 'Neelum', one of the parents of 'Amrapali' and 'Mallika' was included in the study to understand genetic relationship with its hybrids.

48. Samant *et al.*, (2010) investigated genetic diversity among 63 mango genotypes (Mangifera indica L.) was using Inter Simple Sequence Repeat (ISSR) markers. A total of 334 scorable amplification products were detected with 28 ISSR primers of which 331 (99.10%) were polymorphic. Most of the primers (89.29%) exhibited 100% polymorphism. Primers UBC-812 and UBC-891 identified with the highest number of genotypes with unique fingerprints (53). The highest number of different fingerprints (58) was obtained with primer UBC-812, while the lowest number of different fingerprints (17) was obtained with

primer ISSR-5. Primers UBC-812, UBC-891, UBC-808 and UBC-836 were found to be of high value for fingerprinting in mango as they were able to resolve 58, 57, 55 and 55 of 63 mango genotypes selected for the study, respectively. The Jaccard's similarity values ranged from 0.25 (between 'Cambodiana' and 'Mombasa') to 0.79 (between 'K-1' and 'K-3') with a mean of 0.53. UPGMA tree constructed on ISSR data on the basis of Jaccard's similarity coefficient clustered 55 of 63 mango genotypes into six major groups, however eight mango genotypes remained unclustered. The Cluster 1 comprised of mainly south Indian genotypes, while Cluster 2 comprised of mainly man-made hybrids and genotypes from the Northern and Eastern regions of India. Genotypes indigenous to Western parts of India grouped in Cluster 3. Cluster 4 had the exotic genotypes introduced from Brazil, while Clusters 5 and 6 comprised of Floridan mango genotypes. UPGMA clustering of ISSR data showed good correspondence with pedigree, geographical separation and embryo types, i.e. mono- or poly-embryonic.

49. Bhargava *et al.*, (2011) analyzed genetic variation and relationship of mango germplasm using Random Amplified Polymorphic DNA (RAPD) for *Arka Anmol* and *Ratna*. The amplified DNA fragments (amplicons) obtained and compared by agarose gel electrophoresis. Out of many primers screened, one was selected and 5 and 6 bright bands of *Arka Anmol* and *Ratna* were found out, respectively. Out of which 4 bands were shared and others were unique. RAPD is highly polymorphic nature and genetic diversity was studied accordingly. *Arka Anmol* and *Ratna* are very close to each other and showed a minimum

dissimilarity of 1%. The data suggest that RAPD may be of value by virtue of its rapidity, efficiency and reproducibility in generating genetic fingerprints.

50. Gajera *et al.*, (2011) analyzed the genetic variability and relationships among 20 *Mangifera indica* genotypes representing 15 endangered and 5 cultivars, obtained from Indian Gir forest region, by using 10 random amplified polymorphic DNA (RAPD) and 21 inter simple sequence repeat (ISSR) markers. Also, the average numbers of polymorphic loci per primer, average polymorphic information content (PIC) and primer index (PI) values were more for RAPD than for ISSR. But, total number of genotype specific marker loci, Nei's genetic diversity (h), Shannon's information index (I), total heterozygosity (Ht), average heterozygosity (Hs) and mean coefficient of gene differentiation (Gst) were more for ISSR as compared to RAPD markers. The regression test between the two Nei's genetic diversity indexes showed low regression between RAPD and ISSR based similarities but maximum for RAPD and RAPD + ISSR based similarities. The pattern of clustering of genotypes within groups was not similar when RAPD and ISSR derived dendrogram were compared. Thus, both the markers were equally important for genetic diversity analysis in *M. indica*.

51. Samal *et al.*, (2011) undertook an assessment of genetic diversity studies to understand the level and pattern of diversity in 65 mango (*Mangifera indica* L.) genotypes of India including 20 commercial cultivars, 18 hybrids, 25 local genotypes and two exotic cultivars based on qualitative and quantitative fruit characters as well as RAPD and ISSR profiles. A considerable variation was observed in respect of three important qualitative

characters namely table quality, fruit attractiveness and storage life of ripe fruits and potentially superior genotypes for the above traits were identified. Fifteen RAPD primers yielded 27 monomorphic and 129 polymorphic bands with percent polymorphism averaging 82.7%. Of a total 70 ISSR bands generated from eight ISSR primers, 60 bands (85.71%) were found to be polymorphic. UPGMA dendrograms drawn using RAPD, ISSR and cumulative data showed highly similar grouping of genotypes on the basis of their parental origin.

52. Souza *et al.,* (2011) evaluated genetic variability of 35 mango (*Mangifera indica*) accessions maintained in the Active Germplasm Bank of Embrapa Meio-Norte in Teresin, Piauí, Brazil, using RAPDs. Genomic DNA, extracted from leaf material using a commercial purification kit, was subjected to PCR with the primers A01, A09, G03, G10, N05, and M16. Fifty-five polymorphic loci were identified, with mean of 9.16 ± 3.31 bands per primer and 100% polymorphism. The mango accessions were found to have considerable genetic variability, demonstrating the importance of analyzing each genotype in a collection in order to efficiently maintain the germplasm collection.

53. Padwale (2012) assessed the genetic variation in mango (Cv. Alphonso) at various locations in Konkan region. RAPD profile for all Mango plants of various location (Cv. Alphonso) were generated with 38 random decamer primers. Out of 38 primers screened 10 primers produced scorable DNA fragments and each of the 10 random primers revealed polymorphism. These primers generated 201 DNA fragments in the average range of 348.3 bp to 812.2 bp, of which 140 were polymorphic.

The average level of polymorphism generated by the primers was high (67.37%). The primers OPF-20, OPM-12, and OPU-08 produced distinct RAPD patterns (85.71, 83.33 and 76.92% polymorphism) for all the Mango plants. The average discrimination power among the 10 primers was 52.5 per cent. The overall range of the similarity among all Mango samples was found to be very wide, ranging from 0.086 to 0.571 which indicates there was high variability among the Alphonso cultivars under study.

54. Rocha *et al.*, (2012) assessed the genetic diversity of 'Uba ´' mango trees cultivated at the Zona da Mata of Minas Gerais State, Brazil, to identify whether there is variability in the plants grown in the region, justifying the mass selection as a breeding method. They studied 102 accessions. Leaves were collected for extraction of genomic DNA, which was amplified with nine ISSR primers. The data obtained by the analysis of electrophoretic patterns were arranged in a binary matrix, considering 0 for the absence and 1 for the presence of bands. Based on these data, we performed the analysis of genetic dissimilarity and carried out the cluster analysis by the methods of Tocher and graphical dispersion. The most similar accessions are 144 and 150, both coming from Uba ´, while the most divergent ones are 29 and 97, from Visconde do Rio Branco. The grouping by the Tocher method separated the accessions into six groups, 94.1% of which were allocated in the first group and showed that there is no separation of accessions depending on the sampling sites.

55. Vasugi *et al.,* (2012) stated that microsatellites were successfully used for genetic diversity analysis of the indigenous 'Appemidi' type. Also, the major compounds that contribute to

the unique aroma of these types were estimated. The materials used for this study consisted of 43 accessions and 14 SSRs developed at the Indian Institute of Horticultural Research, Bangalore. The analysis of 211 bands detected by the 14 Simple Sequence Repeats (SSRs) markers showed unambiguous discrimination of the 43 mango genotypes. The dendrogram resulted in the grouping of accessions into two major clusters, viz. cluster I with highly acidic types and cluster II with less acidic and high TSS group.

56. Begum *et al.*, (2013) studied the *In-Situ* morphological and *Ex-Situ* microsatellite analysis of fruit and leaf samples of 16 trees of 'Panchadarakalasa' (PK Acc-l to PK Acc-16) spread over the three eco-geographical regions (Coastal Andhra, Rayalaseema and Telangana) of the Andhra Pradesh were collected during summer 2009, which respectively to identify whether there is variability in the plants grown in the A. P. Characterization and evaluation of fruit samples based on 9 quantitative and 7 qualitative traits revealed phenotypic variations among accessions under study. Twenty out of 109 mango-specific microsatellite markers validated, were amplified. Of the 20 microsatellites amplified, only 4 were polymorphic with a total of 11 alleles ranging from 130 bp to 245 bp. The polymorphic information content of the polymorphic alleles ranged from 0.25-0.56, whereas the Jaccard's similarity coefficient values ranged from 0.9-1.0. The pair-wise genetic dissimilarities ranged from 0.00-0.10 with a mean value of 0.05. Microsatellite analysis revealed smaller intracultivar variability of 10% in In-Situ conditions and a genetic divergence between trees attesting that 'Panchadarakalasa' whatsoever cultivated

throughout the state is not pure clone. Highly polymorphic microsatellites like SSR-83, MngSSR-24 and MngSSR-26 were more useful in differentiating the 'Panchadarakalasa' accessions.

57. Kumar *et al.,* (2013) conducted experiment to show genetic variation and investigate inter-relationship between 10 mango genotypes. 20 SSR markers were tested with 10 genotypes: Kalepad, Neelum, Swarnarekha, Alphonso, Rumani, Sendura, Banganapalli, Himayuddin, Mulgoa and Bangalora. Polymerase chain reaction (PCR) amplification of the DNA isolated from 10 mango genotypes with 20 SSR primers produced a total of 240 amplified products, of which 184 were polymorphic and 56 monomorphic. The sizes of the alleles detected ranged from 120 to 369 bp. SSR markers were highly polymorphic with an average of 2.70 alleles per primers. SSRs gave moderate values of polymorphic information content (PIC) range of 0.320 to 0.774. The amplified products varied between 2 (LMMA 1, 5, 7, 12, 16, MiSHRS-1 and MiSHRS-37) to 3 and 4 (LMMA 4, 6, 9, 10, 11, 13, 14, 15 MiSHRS-4, 48, 18, 39 and LMMA 8) bands per primer. They obtained moderate degree of genetic diversity, with Jaccard's similarity co-efficient values ranging from 0.075 between cluster I and II to 0.285 between clusters II and III. The unique fingerprints size ranged from LMMA-8 (257-270 bp), LMMA-11 (232- 245 bp) to MiSHRS 39 (340-369 bp).

58. Archak *et al.,* (2014) were analyzed 23 such popular cultivars of mango belonging to different regions of India by employing multi-locus marker techniques to measure the genetic diversity existing among them. 12 unanchored ISSR primers (114 markers) and 15 AFLP primer-pair combinations

(1,073 markers) revealed average gene diversity over loci to be 0.231 and 0.257 respectively. Mango cultivars from southern India were found to be significantly different ($p<0.001$). In order to decipher how these markers are inherited, three popular hybrids 'Amrapali', 'Mallika' and 'Ratna' were compared with their parents for band sharing information. Number of ISSR markers shared between pairs of parents and their hybrid was nearly 20% more than that of AFLP markers, endorsing the conserved nature of ISSR profiles.

59. Begum *et al.,* (2014) taken an eco-geographic survey covering the three regions of the state, 31 accessions of 'Beneshan' (BN Acc-l to BN Acc-31) were selected and their fruit and leaf samples were collected to study intracultivar heterogeneity based on morphological fruit traits and microsatellite markers, respectively. Out of the 109 mango-specific simple sequence repeats (SSRs) validated, 23 were polymorphic. Polymorphic microsatellites produced a total of 58 alleles, of which 30 were polymorphic (51.72%). The polymorphic information content values varied from 0.03 (SSR-59) to 0.72 (SSR-87). Highly polymorphic microsatellites like SSR-80, SSR-87, SSR-28, and SSR-89 were more useful in differentiating the 'Beneshan' accessions. Microsatellites SSR-91 and MngSSR-26 produced unique alleles of 280 and 140 bp in BNAcc-8 and BNAcc-9 accessions, respectively. Jaccard's similarity coefficient varied from 0.50 to 1.00. There was a wide range of intravarietal heterogeneity (up to 50%).

60. Dillon *et al.*, (2014) studied a collection of 24,840 expressed sequence tags (ESTs) generated from five mango (Mangifera indica L.) cDNA libraries mined for EST-based simple

sequence repeat (SSR) markers. Over 1,000 ESTs with SSR motifs were detected from more than 24,000 EST sequences with di- and tri-nucleotide repeat motifs the most abundant. Of these, 25 EST-SSRs in genes involved in plant development, stress response, and fruit color and flavor development pathways were selected, developed into PCR markers and characterized in a population of 32 mango selections including *M. indica* varieties, and related Mangifera species. Twenty-four of the 25 EST-SSR markers exhibited polymorphisms, identifying a total of 86 alleles with an average of 5.38 alleles per locus, and distinguished between all Mangifera selections. Private alleles were identified for Mangifera species. These newly developed EST-SSR markers enhance the current 11 SSR mango genetic identity panel utilized by the Australian Mango Breeding Program.

61. Tomar *et al.*, (2014) conducted the experiment on 20 mango genotypes found in Gir region of Gujarat state using 21 ISSR primers. The primers yielded a total 125 bands and 119 scorable polymorphic markers, accounting for 95.2% of total reproducible amplification products in the range 42 to 2522 bp. Each primer could amplify 4 to 11 DNA bands, of which primer UBC-840 generated the highest number of bands (11) followed by UBC 855, UBC 835, and UBC 848. While primers UBC 817, UBC 825, UBC 844, UBC 884 and UBC 891 showed the lowest number (4) of DNA bands. Out of the total 119 polymorphic DNA bands, 34 were unique indicating of its presence only in one of the landraces. Jaccard's similarity coefficient between genotypes ranged from 0.21 to 0.59. The maximum genetic

similarity was found between 'Jamadar' and 'Kesar' and lowest was between 'Khodi' and 'Agargato'.

62. Ariffin *et al.*, (2015) employed ISSR markers to reveal genetic diversity and genetic relatedness among 28 Mangifera accessions collected from Yan (Kedah), Bukit Gantang (Perak), Sibuti (Sarawak), and Papar (Sabah). A total of 198 markers were generated using nine anchored primers and one non anchored primer. Genetic variation among the 28 accessions of Mangifera species including wild relatives, landraces, and clonal varieties is high, with an average degree of polymorphism of 98% and mean Shannon index, $H0$ = 7.50. Analysis on 18 *Mangifera indica* accessions also showed high degree of polymorphism of 99% and mean Shannon index, $H0$ = 5.74. Dice index of genetic similarity ranged from 0.0938 to 0.8046 among the Mangifera species. The dendrogram showed that the Mangifera species were grouped into three main divergent clusters. Cluster 1 comprised 14 accessions from Kedahand Perak. Cluster II and cluster III comprised 14 accessions from Sarawakand Sabah. Meanwhile, the Dice index of genetic similarity for 18 accessions of Mangifera indica ranged from 0.2588 to 0.7742. The dendrogram also showed the 18 accessions of *Mangifera indica* were grouped into three main clusters. Cluster I comprised 10 landraces of *Mangifera indica* from Kedah. Cluster II comprised 7 landraces of *Mangifera indica* followed by Chokanan to form Cluster III.

63. Dinesh *et al.*, (2015) evaluated seedling diversity for morphological traits in the Chittoor area of Andhra Pradesh in India. The statistical analysis carried out for fruit characteristics showed significant differences among the

varieties for various fruit characteristics. Molecular characterization was carried out using microsatellite markers. Most of the indigenous varieties from Kalepalli region are grouped in the same cluster. The 8-labeled SSR markers employed to study detected 97 alleles in 44 genotypes. The number of alleles for each locus ranged from 9 to 19, with a mean number of alleles per locus being 12.13. Observed heterozygosity (Ho) and expected heterozygosity (He) for each locus ranged from 0.364 to 0.674 and 0.693 to 0.889, respectively.

64. Ravishankar *et al.*, (2015) assessed the genetic diversity and population structure of mango cultivars by employing 14 simple sequence repeat markers, with high polymorphic information content. A set of 387 mango accessions from different regions of India was used. Model based structure analysis revealed the presence of two subpopulations comprising the cultivars from 'South-West' region and 'North-East' region. A similar clustering pattern was observed in the dendrogram analysis, with two major groups identified that were further sub-grouped based on their genetic relatedness. Analysis of molecular variance showed a significant variance component among and within mango sub-populations derived from the structure analysis. The proportion of genetic differentiation among individuals within the two populations was found to be significant with a FST value of 0.248.

65. Sherman *et al.*, (2015) conducted the study on diversity in mango collections with a small number of genetic markers. They described a de novo transcriptome assembly from mango cultivar 'Keitt'. Variation discovery was performed using

Illumina resequencing of 'Keitt' and 'Tommy Atkins' cultivars identified 332,016 single-nucleotide polymorphisms (SNPs) and 1903 simple-sequence repeats (SSRs). Most of the SSRs (70.1 %) were of trinucleotide with the preponderance of motif (GGA/AAG)n and only 23.5 % were di-nucleotide SSRs with the mostly of (AT/AT)n motif. Further investigation of the diversity in the Israeli mango collection was performed based on a subset of 293 SNPs. Those markers have divided the Israeli mango collection into two major groups: one group included mostly mango accessions from Southeast Asia (Malaysia, Thailand, and Indonesia) and India and the other with mainly of Floridian and Israeli mango cultivars. The latter group was more polymorphic (FS =−0.1 on the average) and was more of an admixture than the former group. A slight population differentiation was detected (FST=0.03).

66. Kheshin *et al.,* (2016) characterized and estimated genetic polymorphism and relationships among five mango accessions (collected from different governorates) based on ISSR markers. 78 morphological characteristics were studied to describe the fruit and stone. Inter-simple sequence repeats (ISSR) markers were used to study the genetic diversity and phylogenetic relationships among the collected accessions. The 12 ISSR primers produced a total number of amplified bands ranged from 6 to 19 fragments. The highest number of fragments was 19 bands for $(TC)_8GT$ primer. While, $(CA)_6GT$ primer generated the lowest number of amplicons (6 bands). The average number of fragments/primer was (11.25) and the size of these fragment ranged from 326-3125 bps. The percentage of polymorphism

revealed by the different primers ranged from 7.14 to 66.67 % with average of 42.86%.

67. Pruthvish *et al.*, (2016) studied the genetic diversity and relationships among mango varieties using RAPD molecular markers. RAPD was employed to study the genetic diversity and Inter-relationship among 19 Mango varieties. On an average RAPD analysis generated 5-6 discrete bands/ varieties with 10 nucleotides primers. The size of the amplified products ranged from 100-3500 base pairs in length. With an average of 5-10 bands per primer. Of 21 amplified fragments 50 were polymorphic (80%) with at least one pair wise comparison between 19 varieties.

68. Galal *et al.*, (2017) studied the genetic variability and molecular characterization of some local and imported mango cultivars in Egypt. The investigation was conducted to assess the genetic variability and heritability for some physico-chemical traits of some major local and imported mango cultivars grown in Egypt. Moreover, study of the genetic divergence and phylogenic relationships; based on RAPD markers, among the genotypes studied to provide bases for marker-assisted selection of parents for hybridization and improvement of mango cultivars.

69. Thakur *et al.*, (2017) studied molecular characterization of Mango by adopting RAPD-PCR markers. A total of 99 amplicon levels were produced by 4 primers available for analysis. The highest number of 24 amplicon levels was produced by OPK-04 followed by 23 in OPK-06 and the least of two marker levels was produced by OPB -01. Out of total 99 scorable bands 2 unique bands were produced by primer namely OPB-01. The presence

of high genetic diversity (71%) indicates that the population has plenty of scopes for breeding program.

CHAPTER III

MATERIAL AND METHODS

The detailed information regarding the materials used and methods followed during the course of the present investigation is mentioned in this chapter. The investigations were carried out in the laboratory of Plant Biotechnology Centre, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist. Ratnagiri (M.S.)

Experiment Details:-

3.1 Source of Plant Material:

For the present study, the experimental study leaf samples of the mango cultivar Alphonso were collected from the following different locations and mentioned in Table 2.

Table 2: Details of 50 leaf samples used in the study.

Sr. No.	Name of farmer & Location	Sample No.	North Latitude	East Longitude
1.	Dr. Vishwas Ashok Kelkar **Ambivali–Kelshi** Tal. Dapoli Dist. Ratnagiri	K1	17°90'78.12''	73°08'04.91''
		K2	17°90'82.41''	73°08'04.52''
		K3	17°90'78.64''	73°08'18.14''
		K4	17°90'78.65''	73°08'17.74''
		K5	17°90'78.57''	73°08'17.41''
2.	Dr. Makrand Shrinivas Joshi **Murud** Tal. Dapoli Dist. Ratnagiri	M1	17°77'31.62''	73°11'89.24''
		M2	17°77'35.80''	73°11'97.96''
		M3	17°46'60.96''	73°90'52.46''
		M4	17°46'80.51''	73°75'52.62''
		M5	17°46'20.41''	73°71'60.42''
3.	Mr. Sunil Godbole **Pawas** Tal. Ratnagiri Dist. Ratnagiri	P1	16°52'55.74''	73°19'21.52''
		P2	16°53'15.68''	73°19'80.76''
		P3	16°52'56.01''	73°19'23.26''
		P4	16°52'56.51''	73°19'23.66''
		P5	16°52'56.18''	73°19'21.04''
4.	Mr. Surendra Shridhar Karekar **Adivare** Tal. Rajapur Dist. Ratnagiri	A1	16°43'12.43''	73°20'38.56''
		A2	16°43'12.26''	73°20'39.40''
		A3	16°43'12.48''	73°21'80.15''
		A4	16°43'10.95''	73°20'40.33''
		A5	16°43'11.21''	73°20'40.33''
5.	Mango Research Sub Centre, **Rameshwar-Girye** Tal. Deogad Dist. Sindhudurg. Dr. B. S. K. K. V. Dapoli.	G1	16°31'40.51''	73°20'43.10''
		G2	16°31'40.39''	73°20'12.44''
		G3	16°31'39.71''	73°20'44.78''
		G4	16°31'39.48''	73°20'45.44''
		G5	16°31'39.84''	73°20'45.33''
6.	Mr. Kiran Manohar Marathe **Padel** Tal. Deogad Dist. Sindhudurg.	D1	16°28'22.10''	73°23'40.42''
		D2	16°28'41.79''	73°22'34.36''
		D3	16°28'21.98''	73°23'40.08''
		D4	16°28'21.79''	73°23'40.78''
		D5	16°28'21.79''	73°23'40.74''
7.	Mr. Prasanna Gogate	J1	16°26'30.54''	73°24'60.80''

	Nadan–Jamsande Tal. Deogad Dist. Sindhudurg.	J2	16°26'29.45''	73°26'62.70''
		J3	16°26'30.23''	73°24'61.12''
		J4	16°26'31.56''	73°24'61.43''
		J5	16°26'30.75''	73°24'61.69''
8.	Mr. Mahesh Madhukar Rane **Achara** Tal. Malvan Dist. Sindhudurg.	C1	16°12'44.12''	73°28'51.45''
		C2	16°12'51.46''	73°28'50.23''
		C3	16°12'36.63''	73°28'53.66''
		C4	16°12'51.23''	73°28'53.81''
		C5	16°12'51.79''	73°28'52.92''
9.	Regional Fruit Research Station (RFRS), **Vengurle**. Tal. Vengurle Dist. Sindhudurg. Dr. B. S. K. K. V. Dapoli.	V1	15°52'48.85''	73°38'66.12''
		V2	15°52'60.64''	73°38'59.53''
		V3	15°52'55.19''	73°38'48.87''
		V4	15°52'36.20''	73°38'87.66''
		V5	15°52'72.43''	73°38'59.90''
10.	Mr. Satish Wanjari **Katta** Tal. Malvan Dist. Sindhudurg.	L1	15°52'21.55''	73°39'29.55''
		L2	15°52'20.33''	73°39'33.28''
		L3	15°52'20.45''	73°39'54.92''
		L4	15°52'23.91''	73°39'33.50''
		L5	15°52'20.42''	73°39'31.74''

All leaf samples were frozen in ice bags for transportation to the laboratory and subsequently stored at below 0°C until processed.

3.2 Laboratory Resources and Techniques

The entire laboratory work was done in the laboratory of Plant Biotechnology Centre, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth Dapoli, Dist- Ratnagiri (M. S.)

3.2.1 Extraction of genomic DNA

3.2.1.1 Plant material

For the present experimental study, young newly flushing leaves from the selected trees were used for the extraction of genomic DNA.

3.2.1.2 Stock solutions prepared

The various solutions, buffers and its concentration used for extraction of the DNA are mentioned as under.

a) Extraction buffer stock solutions:

Table 3: Detail of components used for preparation of working buffer stock solutions:

Sr. No.	Chemicals	Quantity required for 100 ml (g)
1.	200 mM Tris-HCl	2.423
2.	25 mM EDTA	0.831
3.	250 mM NaCl	1.461

b) Composition of Extraction Buffer

Quantity of chemicals required for preparation of 10 ml working extraction buffer was standardized by taking three different levels i.e. T_1, T_2 and T_3 (Table 4). The chemical levels which produced good quality as well as quantity of DNA were used for further study.

Table 4: Quantity of components

Sr. No.	Components	T_1	T_2	T_3
1.	Glucose (0.5M)	0.800 g	0.900 g	1.000 g
2.	PVP (3%)	0.050 g	0.100 g	0.150 g
3.	Sodium Bisulphite (0.4%)	0.030 g	0.040 g	0.050 g
4.	Sodium Lauryl Sulphate (0.5%)	0.040 g	0.050 g	0.060 g
5.	Sarcosyl (5%)	400 µl	500 µl	600 µl
6.	Working buffer (Tris- HCl, EDTA & NaCl)	10 ml	10 ml	10 ml

c) Chloroform Isoamyl alcohol mixture (24:1)

d) 100% Chilled Isopropanol

e) 70% Ethyl alcohol

f) 1X TE Buffer

3.2.1.3 Procedure for extraction of genomic DNA

The DNA was isolated by following the protocol of Doyle and Doyle (1990) i.e. Rapid method with slight modifications of buffer composition and concentration.

The young newly emerged leaves were collected and sterilized with 70% ethanol to avoid the contamination. The extraction of genomic DNA was done using the following protocol:

1. Leaf tissue (100 mg) was collected in a 1.5 ml eppendorf tube which leads to ensure uniform size of sample.
2. Collected tissue was macerated by micro pestle at room temperature without buffer for 15 sec.
3. Extraction buffer (500 µl) was added and leaf tissue macerated gently for few seconds and kept in hot water bath for 45 minutes at 65°C.
4. The sample was cooled down at room temperature and centrifuged at 8000 rpm, at 4°C for 10 minutes.
5. Aqueous layer was transfered to fresh eppendorf tube and 200 µl of C: IA (24:1) was added and mixed by gentle inversion for 5-6 times. The contents were then centrifuged at 8000 rpm, at 4°C for 10 minutes.
6. Supernatant was mixed with 2/3 of chilled isopropanol and incubated at –20°C overnight.
7. On the next day, the solution was centrifuged at 8000 rpm, at 4°C for 10 minutes and pellet was collected.

8. Pellet was washed with 100 µl of 70 per cent ethanol followed by centrifugation at 8000 rpm, at 4°C for 10 minutes.
9. Pellet was dried and re-suspended in 50 µl of 1X TE buffer.
10. Incubated at 37°C in a water bath for 30 minutes and stored at -20°C till further use.

3.2.2 DNA Purification

Purification of DNA was done to remove RNA and proteins which were the major contaminants. RNA was removed by RNase treatment and proteins were removed by Proteinase K treatment.

3.2.3 DNA Quantification by using Agarose gel electrophoresis

Concentration of DNA in the sample was determined by agarose gel electrophoresis with standard DNA i.e. DNA Ladder on 0.8 per cent agarose gel and by comparison of the intensity of band staining with Ethidium Bromide.

3.2.4 Dilution of Crude extracts of DNA

The extracted DNA from all the samples was in varying concentrations. While preparing the working DNA samples it was diluted in such a manner as detailed in Table 5, so as it maintains the DNA content in all the samples in similar concentration.

Table 5: **Sample wise dilution of the extracted DNA**

Sr. No.	Dilution Ratio	Quantity of DNA (µl)	Quantity of D/W (µl)
1.	1:10	1	10
2.	1:20	1	20
3.	1:30	1	30
4.	1:40	1	40
5.	1:50	1	50
6.	1:60	1	60

7.	1:70	1	70
8.	1:80	1	80
9.	1:90	1	90
10.	1:100	1	100

3.2.5 DNA Amplification

The isolated and quantified DNA sample was subjected to DNA amplification by the use of following components.

3.2.5.1 Requirements

a) ISSR primers: A set of 40 ISSR primers (Table 6) composed wholly of defined, short tandem repeat sequences with anchor, and representing different microsatellites (di and tri-repeats) have been used as generic primers in PCR amplification of inter simple sequence repeat regions as per the method of Adawy *et al.*, (2004).

b) Template DNA: Purified DNA from Alphonso leaf sample.

c) dNTPs: dNTPs mixture of 2.5 mM each obtained from HiMedia Laboratories Pvt. Ltd., Mumbai- 400 086 (M. S.)

d) *Taq* polymerase: 3.0 U/µl *Taq* DNA polymerase each obtained from HiMedia Laboratories Pvt. Ltd., Mumbai- 400 086 (M. S.)

e) Taq buffer: Taq buffer 10X obtained from HiMedia Laboratories Pvt. Ltd., Mumbai- 400 086 (M. S.)

f) $MgCl_2$: $MgCl_2$ mixture of 25 mM each obtained from Bangalore Genei Pvt. Ltd., Banglaore.

g) Thermal cycler: Eppendorf, Master cycler gradient supplied by Eppendorf gradient, 2231, Hamburg Germany was used for cyclic amplification of DNA.

Table 6: List of ISSR primers with their sequence.

Sr.	Primer	Primer sequence	GC	Tm

No.			Content	Value
		(5’ – 3’)	(%)	(^{0}C)
1.	UBC-807	AGA GAG AGA GAG AGA GT	47.1	45.0
2.	UBC 811	GAG AGA GAG AGA GAG AC	52.9	43.3
3.	UBC 812	GAG AGA GAG AGA GAG AC	52.9	44.4
4.	UBC 813	CTC TCT CTC TCT CTC TT	47.1	43.5
5.	UBC 814	CTC TCT CTC TCT CTC TA	47.1	41.4
6.	UBC-815	CTC TCT CTC TCT CTC TG	52.9	45.0
7.	UBC-816	CAC ACA CAC ACA CAC AT	47.1	51.2
8.	UBC-817	CAC ACA CAC ACA CAC AA	47.1	52.8
9.	UBC-818	CAC ACA CAC ACA CAC AG	52.9	52.1
10.	UBC-824	TCT CTC TCT CTC TCT CG	52.9	49.1
11.	UBC-825	ACA CAC ACA CAC ACA CT	47.1	49.3
12.	UBC-831	ATA TAT ATA TAT ATA TYA	5.55	20.2
13.	UBC-833	ATA TAT ATA TAT ATA TYG	11.1	55.0
14.	UBC-834	AGA GAG AGA GAG AGA GT	50.0	49.8
15.	UBC-841	GAG AGA GAG AGA GAG AC	55.5	45.7
16.	UBC-843	CTC TCT CTC TCT CTC TRA	50.0	37.6
17.	UBC-844	CTC TCT CTC TCT CTC TRC	55.5	39.4
18.	UBC-845	CTC TCT CTC TCT CTC TRG	55.5	43.4
19.	UBC-852	TCT CTC TCT CTC TCT CRA	50.0	44.9
20.	UBC-853	TCT CTC TCT CTC TCT CRT	50.0	54.0
21.	UBC-854	TCT CTC TCT CTC TCT CRG	55.5	51.1
22.	UBC-857	ACA CAC ACA CAC ACA CCG	55.5	57.1
23.	UBC-867	GGC GGC GGC GGC GGC GGC	100	88.6
24.	UBC-869	GTT GTT GTT GTT GTT GTT	33.3	51.0
25.	UBC-871	TAT TAT TAT TAT TAT TAT	00.0	32.2
26.	UBC-872	GAT AGA TAG ATA GAT A	25.0	28.9
27.	UBC-874	CCC TCC CTC CCT CCCT	75.0	33.0
28.	UBC-876	GAT AGA TAG ACA GAC A	37.5	36.4
29.	UBC-878	GGA TGG ATG GAT GGAT	50.0	29.0
30.	UBC-879	CTT CAC TTC ACT TCA	40.0	42.2

31.	UBC-881	GGG TGG GGT GGG GTG	66.6	66.5
32.	UBC-884	HBH AGA GAG AGA GAG AG	47.1	35.0
33.	UBC-885	HBH AGA GAG AGA GAG AG	52.9	41.7
34.	UBC-886	VDV CTC TCT CTC TCT CT	52.9	36.9
35.	UBC-889	DBD ACA CAC ACA CAC AC	47.1	39.4
36.	UBC-891	AGA TGT GTG TGT GTG TG	47.1	51.8
37.	UBC-893	NNN NNN NNN NNN NNN	-	42.0
38.	UBC-894	TGG TAG CTC TTG ATC ANN	-	62.4
39.	UBC-897	CCG ACT CGA GNN NNN NAT	-	56.2
40.	UBC-898	GAT CAA GCT TNN NNN NAT	-	47.3

B = (C, G, T) (i.e. not A) H = (A, C, T) (i.e. not G)
V = (A, C, G) (i.e. not T) D = (A, G, T) (i.e. not C)
Y = C OR T (i.e. not A, G) R = A OR G (i.e. not C, T)

Single letter abbreviations for mixed base positions.

3.2.5.2 Stock solutions

a) 10X buffer

b) $MgCl_2$ (25 mM)

c) ISSR primers (5 mM/µl)

d) 40 ng μl^{-1} Template DNA

e) 3.0 U μl^{-1} *Taq* DNA polymerase.

3.2.5.3 Preparation of master mix

Initially the PCR master mix was standardized by changing the quantity of each component and the optimum concentration of each component in master mix which gave better amplification was chosen for amplification purpose. Quantity of chemicals required for preparation of 20 ml master mixture was standardized by taking three different levels i.e. T_1, T_2 and T_3 (Table 7). The master mix was distributed to 9 tubes and template DNA of the respective sample was added to make the total reaction volume to 20 µl and the mixture was given a short spin to mix the content.

Table 7: Master mixture for PCR (20 µl/tube)

Components	Quantities in µl		
	T1	**T2**	**T3**
Taq buffer (10X)	2.5	2.5	2.5
$MgCl_2$ (25 mM)	0.2	0.5	1.0
dNTPs (10 mM)	0.5	1.0	1.5
Primer (5 mM/µl)	0.5	1.0	1.5
D/W	15.5	13.5	11
DNA (40 ng/µl)	0.5	1.0	1.5
Taq Polymerase (3.0 U/µl)	0.3	0.5	1.0
Total	**20**	**20**	**20**

3.2.5.4 Standardization of Thermo Profile of PCR

Initially the thermo profile of PCR was standardized by changing the temperature of each step in PCR cycle i.e. initial denaturation, denaturation, annealing, initial primer extension and final extension. Three different reaction conditions were tested and the reaction condition which gave better amplification was chosen for amplification purpose (Table 8).

3.2.5.5 Thermal cycling

1. Sterile micro centrifuge tubes were numbered from 1 to 50.
2. Template DNA from individual sample was added to each tube.
3. Master mix was added to all the tubes and was given short spin to mix the contents.
4. The tubes were placed in the thermal cycler for 30 cycles of PCR. Samples were held at 4°C, in the thermal cycler, until the contents were loaded on to the gel for electrophoresis.

Table 8: Thermo profile of PCR.

Sr. No.	PCR steps	Reaction condition		
		I	**II**	**III**
1.	Initial denaturation	94°C for	94°C for	94°C for 5

		5 min.	5 min.	min.
2.	Denaturation	94°C for 20 sec.	94°C for 30 sec.	94°C for 1 min.
3.	Annealing	50°C for 45 sec.	50°C for 1 min.	50°C for 2 min.
4.	Initial primer extension	72°C for 45 sec.	72°C for 1 min.	72°C for 2 min.
5.	Final extension	72°C for 7 min.	72°C for 7 min.	72°C for 7 min.
6.	Hold	4°C	4°C	4°C

3.2.5.6 Standardization of annealing temperature

The standard ISSR markers purchased with concentration of 5 pmol/µl and were employed for optimization of annealing temperature. Various ranges of temperature were adjusted in eppendorf PCR machine following gradient PCR. The reaction mixture was prepared as usual and template DNA was utilized for optimization of annealing temperature.

3.2.5.7 Separation of amplified product by agarose gel electrophoresis

The various chemicals and instruments used for agarose gel electrophoresis of amplified DNA samples are mentioned as under.

3.2.5.7.1 Requirement

a) Electrophoresis unit (Gel casting tray, gel comb, power pack)

b) Gel documentation system

c) Agarose

d) Tracking dye (Bromophenol blue)

e) Ethidium Bromide (1 µg ml^{-1})

f) 50X TAE buffer.

3.2.5.7.2 Procedure

The amplified products in ISSR reaction were separated by electrophoresis in 2 per cent agarose gel (SRL, India), containing Ethidium Bromide in 1X TAE Buffer (pH 8.0) and separation were carried out by applying constant voltage of 100 volts for 90 mins. The standard DNA ladder used was 100 bp and high range DNA ruler. PCR and gel electrophoresis were carried out two times and only reproducible patterns were used for data analysis.

3.2.5.8 Photography and Gel documentation

The agarose gel was photographed under UV light using Pentax K 312 nm camera. The images of gel were also taken by the documentation systems (Uvi-Tech. Fire reader, Cambridge, England) and saved in computer for further analysis.

3.2.6 Statistical analysis

ISSR markers across the 50 samples were scored for their presence (1) or absence (0) of bands for each primer. The binary data so generated was used to estimate the levels of polymorphism by dividing the number of polymorphic bands by the total number of scored bands. Jaccard's similarity coefficients for each pairwise comparison between samples were calculated and similarity co-efficient matrix was generated. This matrix was subjected to unweighted pair group method with arithmetic mean (UPGMA) to construct a dendrogram. The similarity co-efficient analysis and dendrogram construction were carried out by using MVSP-A Multivariate Statistical Package-5785 (Version 3.1).

Distance matrix and dendrogram was constructed based on diversity coefficient generated from pooled data by using unweighted pair group method of arithmetic means (UPGMA), a computer

programme for distance estimation. Other parameters computed were,

$$\text{Per cent polymorphism (\%)} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \text{ X } 100$$

3.2.7 Polymorphic Information Content

Polymorphic information content (PIC) value were calculated as per formula developed by Powell *et al.,* (1996)

$$\mathbf{PIC = 1 - \sum P_{ij}^{2}}$$

Where,

P_{ij} is the frequency of i^{th} and j^{th} locus, summed across the entire locus over all lines.

PIC values range from 0 (monomorphic) to 1 (very highly discriminative, with many alleles each in equal and low frequency) were estimated for each profile generated across 50 alphonso samples.

CHAPTER IV

EXPERIMENTAL RESULTS

The experimental results obtained in the present study of molecular analysis of mango cv. Alphonso samples by using molecular markers are presented in this chapter under the different headings.

4.1 DNA Extraction

4.1.1 Standardization of concentration of chemical constituents

Total of 50 samples were selected for extraction of genomic DNA from alphonso samples which were labelled in order from K1 to L5. Genomic DNA was isolated by rapid DNA method. The quantities of components for 10 ml of extraction buffer used for DNA extraction are given in Table 9.

Table 9: Standardization of concentration of chemical constituents in extraction buffer

Sr. No.	Components	T_1	T_2	T_3
1.	Glucose	0.800 g	**0.900g**	0.950 g
2.	PVP	0.050 g	**0.100g**	0.150 g
3.	Sodium Bisulphite	0.030 g	**0.040g**	0.050 g
4.	Sodium Lauryl Sulphate	0.040 g	**0.050g**	0.060 g
5.	Sarcosyl (5%)	400µl	**500µl**	600µl

Gel photograph of DNA isolated from total 50 samples of Alphonso proved that combination of 0.900 g glucose, 0.100 g PVP, 0.040 g Sodium bisulphite, 0.050 g sodium Lauryl Sulphate and 500 µl Sarcosyl (T_2) were ideal concentration and showed clear DNA bands which were utilized for further process of DNA extraction (Plate 1).

4.1.2 Modifications in the extraction procedure of DNA isolation

The DNA extraction was carried out by the process followed by Doyle and Doyle (1990) with certain modifications. The changes made in various steps are mentioned in the Table 10.

Table 10: Optimization of DNA isolation protocol in Alphonso

Sr. No.	Doyle & Doyle (1990)	Modification	Results
1)	Sample size: 500 mg-1.0 g	Sample size: 200 mg - 500 mg	Reduced size provided less mucilage
2)	Volume of buffer used: Not mentioned	Volume of buffer used: 0.5 ml/sample	Key for good precipitation
3)	Use of polyvinyl polypyrrolidone: No	Use of polyvinyl polypyrrolidone: Yes	Eliminated polyphenol impurities
4)	Incubation Temp: 60 °C for 30 min	Incubation Temp: 65 °C for 45 min	Precipitation of major impurities was observed due to increase in incubation time and temperature
5)	Isopropanol volume used: 0.6 volume.	Isopropanol volume used: twice volume.	Provide good precipitation of DNA under low temperature
6)	Washing: 76 % ethanol	Washing: 70 % ethanol	Maximum removal of salts & purification of DNA

The clear DNA bands observed in gel photograph revealed that the sample size of 200-500 mg was suitable, as it reduces the content of mucilage, which hampers the quality of DNA. The volume of buffer of 0.5 ml/sample yielded good DNA precipitation. Use of PVP was beneficial in removal of polyphenols. The increased temperature of

65°C and time 45 min. resulted in precipitation of major impurities. The use of isopropanol twice the volume in the extraction procedure provided good precipitation of DNA. 70 per cent ethanol was used to remove maximum salts and helped in precipitation of DNA (Plate 1).

4.2 Dilution of Crude extracts of DNA

The extracted DNA from all the samples was in varying concentrations. While preparing the working DNA samples it was diluted in such a manner as detailed in Table 11, so as it maintains the DNA content in all the samples in similar concentration (Table 11).

Table 11: Sample wise dilution of the extracted DNA

Sr. No.	Samples	Dilution Ratio	Quantity of DNA (µl)	Quantity of D/W (µl)
1.	K1	1:20	2	40
	K2	1:20	2	40
	K3	1:20	2	40
	K4	1:20	2	40
	K5	1:20	2	40
2.	M1	1:20	2	40
	M2	1:20	2	40
	M3	1:20	2	40
	M4	1:20	2	40
	M5	1:20	2	40
3.	P1	1:100	2	200
	P2	1:100	2	200
	P3	1:100	2	200
	P4	1:100	2	200
	P5	1:100	2	200
4.	A1	1:100	2	200
	A2	1:100	2	200
	A3	1:100	2	200
	A4	1:100	2	200
	A5	1:100	2	200
5.	G1	1:50	2	100
	G2	1:30	2	60
	G3	1:30	2	60

	G4	1:30	2	60
	G5	1:30	2	60
6.	D1	1:30	2	60
	D2	1:30	2	60
	D3	1:80	2	160
	D4	1:30	2	60
	D5	1:30	2	60
7.	J1	1:80	2	160
	J2	1:100	2	200
	J3	1:100	2	200
	J4	1:100	2	200
	J5	1:100	2	200
8.	C1	1:100	2	200
	C2	1:100	2	200
	C3	1:50	2	100
	C4	1:100	2	200
	C5	1:100	2	200
9.	V1	1:50	2	100
	V2	1:100	2	200
	V3	1:50	2	100
	V4	1:50	2	100
	V5	1:50	2	100
10.	L1	1:30	2	60
	L2	1:30	2	60
	L3	1:30	2	60
	L4	1:30	2	60
	L5	1:30	2	60

The above dilutions were effective as it gave amplifiable quantity of DNA from all the samples of alphonso.

4.3 Stan

dardization of the PCR parameters for rapid method

Slight modifications in PCR parameters like master mixture and thermo profile provides good banding pattern. Modifications in PCR master mixture and thermo profile for ISSR markers which gives better amplification are given in Table 12 and 13.

Table 12: Standardization of the master mixture for ISSR markers

Sr. No.	Chemical contents	Tested range	Remarks
1)	$MgCl_2$ (25 mM)	0.2, 0.5, 1.0 µl	**0.5 µl**: Excess/lower increases the non-specificity and yield of the product
2)	dNTPs (10 mM)	0.5, 1.0, 1.5 (µM)	**1.0 µl**: Provided clear bands
3)	Template DNA (40 ng/µl)	0.5, 1, 1.5 µl	**1 µl**: Reduced pipetting error and gives good amplification
4)	Taq polymerase (3 U/µl)	0.3, 0.5, 1.0 µl	**0.5 µl**: Provided better and clear amplifications.
5)	PCR reaction volume (µl)	10, 20, 25 µl	**20 µl:** Excellent yield of amplicons

Table 13: Standardization of the thermo profile for ISSR markers

Sr. No.	Temperature	Tested range	Remarks
1)	Initial denaturation time 94°C (min)	4, 5, 6 min	**5 min** showed better initial denaturation time
2)	Denaturation time 94°C(sec)	20, 30, 60 sec.	**30 sec** showed better denaturation time
3)	Annealing temperature (°C)	0.45 , 1, 2 min.	**1 min.** showed better annealing time
4)	Initial primer extension	0.45, 1, 2 min.	**1 min.** showed better Initial primer extension time
5)	Final extension	5, 6, 7 min.	**7 min.** showed better final extension time

4.4 Standardization of annealing temperature

Various ranges of temperature were adjusted in PCR machine by following gradient PCR. The reaction mixture was prepared as per the procedure and template DNA was utilized for optimization of annealing temperature. The standard annealing temperature range for ISSR markers was 40.0 to 54.8°C. The standardized annealing temperatures of ISSR markers are given in Table 14.

Table 14: Standardization of annealing temperatures of ISSR marker

Sr. No.	Name of primer	Tm value (°C)	Temperature range (°C)	Standardized Annealing temperature (°C)
1.	UBC 811	43.3	40-50	43.4
2.	UBC 812	44.4	45-55	52.0
3.	UBC 815	45.0	40-50	49.5
4.	UBC 817	52.8	40-50	47.0
5.	UBC 818	52.1	45-55	47.9
6.	UBC 834	49.8	45-55	50.4
7.	UBC 853	54.0	45-55	54.4
8.	UBC 854	51.1	45-55	54.8
9.	UBC 857	57.1	45-55	51.7
10.	UBC 876	36.4	40-50	40.0
11.	UBC 881	66.5	45-55	50.0
12.	UBC 884	35.0	40-50	40.0
13.	UBC 885	41.5	40-50	40.7
14.	UBC 886	36.9	45-55	51.4
15.	UBC 889	39.4	45-55	47.0
16.	UBC 891	51.8	45-55	50.0

4.5 Marker analysis

4.5.1 ISSR analysis in between selected ten locations

The ISSR profile of ten samples of alphonso from selected ten locations were computed individually for each primer and were used for further analysis. The following individual samples from selected 10 locations were taken as a representative sample i.e. C1 from Achara, A1 from Adivare, G1 from Rameshwar-Girye, J1 from Nadan-Jamsande, L1 from Katta, K1 from Ambivali- Kelshi, M1 from Murud, D1 from Padel, P1 from Pawas and V1 from Vengurle were

taken to assess the genetic variation present in between selected 10 locations. The ISSR pattern of genomic DNA of these 10 samples were analyzed with respect to the fragments, informativeness of the markers and polymorphism for the assessment of genetic diversity present in between selected 10 locations.

4.5.1.1 Per cent polymorphism in selected ten locations

The primer wise amplification detail of the genomic DNA of selected 10 locations and per cent polymorphism across the 16 ISSR primers is presented in the Table 15. A total of 746 scorable DNA fragments were produced and among them 326 DNA fragments were found to be polymorphic. The minimum number (10) of polymorphic fragments produced by UBC-889 primer, while the maximum number of polymorphic fragments were found to be 38 (UBC-815 and UBC-854). The average per cent polymorphism across the 16 primers in between 10 samples of selected locations found to be 46.62 per cent. The lowest polymorphism percentage (10 %) was shown by the primer UBC-889 whereas highest polymorphism percentage (100 %) was observed in the primer UBC-815, UBC-817, UBC-853, UBC-857 and UBC-884. The primer UBC-891 produced monomorphic banding pattern. The product size ranged from 0.320 Kb to 2.891 Kb (UBC-885 and UBC-854) (Table 15).

4.5.1.2 Genetic Distance in between selected ten locations

The genetic distance was computed considering all the five varieties from the pooled data and the dendrogram was constructed. The distance similarity matrix is based on Jaccard's similarity coefficient is presented in Table 16. The overall range of the similarity in between ten samples of alphonso was 0.559 to 0.733. The maximum similarity coefficient was observed between Nadan-

Jamsande and Katta (0.733), while lowest similarity coefficient was observed between Padel and Pawas (0.559) (Table 16).

4.5.1.3 Cluster analysis of selected 10 locations of Alphonso

The dendrogram (Fig. 1) based on Jaccard's similarity coefficient was constructed using UPGMA after analysis of banding patterns generated by 10 Alphonso samples from 10 locations with 16 ISSR primers. The dendrogram separated locations into two main clusters, *viz*; I and II. Cluster I contains only one location i.e. Pawas. Cluster II divided into two sub clusters, *viz*; IIA and IIB. IIA contains Ambivali-Kelshi and Murud. IIB again sub divided into two clusters IIBa and IIBb. IIBa contains Padel and Rameshwar-Girye while IIBb consists remaining all locations i.e. Achara, Adivare, Nadan-Jamsande, Katta, Vengurle (Table 17).

Table 15: ISSR primer wise amplification and percent polymorphism in between selected 10 locations of Alphonso.

Sr. No.	Primer	Total No. Bands	Total No. of 0Polymorphic Bands	% Polymorphism	Range of Amplification (Kb)
1	UBC-811	62	22	35.48	0.440-1.994
2	UBC-812	51	11	21.56	0.412-1.109
3	UBC-815	38	38	100.0	0.776-2.410
4	UBC-817	20	20	100.0	0.922-1.108
5	UBC-818	65	15	23.07	0.490-2.224
6	UBC-834	31	21	67.74	0.780-1.458
7	UBC-853	20	20	100.0	0.466-1.116
8	UBC-854	48	38	79.16	0.388-2.891
9	UBC-857	19	19	100.0	0.379-0.510
10	UBC-876	72	32	44.44	0.623-2.182
11	UBC-881	59	19	32.20	0.423-1.998
12	UBC-884	19	19	100.0	1.500-1.575
13	UBC-885	54	24	44.44	0.320-1.335
14	UBC-886	68	18	26.47	0.408-2.086
15	UBC-889	50	10	20.00	0.566-0.978
16	UBC-891	70	0	00.00	0.462-1.474
	Total	**746**	**326**	**-**	**-**
	Average	**46.62**	**21.73**	**46.62**	**0.584-1.649**

Table 16: Genetic similarity coefficient based on ISSRs pooled over the 16 primers in ten samples of alphonso from selected ten locations.

	Achara	Adivare	Rameshwar-Girye	Nadan-Jamsande	Katta	Ambivali-Kelshi	Murud	Padel	Pawas	Vengurle
Achara	1.000									
Adivare	0.711	1.000								
Rameshwar-Girye	0.678	0.693	1.000							
Nadan-Jamsande	0.719	0.716	0.721	1.000						
Katta	0.670	0.724	0.709	0.733	1.000					
Ambivali-Kelshi	0.606	0.674	0.678	0.682	0.709	1.000				
Murud	0.566	0.594	0.667	0.634	0.697	0.685	1.000			
Padel	0.591	0.659	0.723	0.667	0.655	0.625	0.652	1.000		
Pawas	0.594	0.641	0.591	0.596	0.620	0.609	0.600	0.559	1.000	
Vengurle	0.628	0.716	0.626	0.705	0.722	0.591	0.617	0.648	0.667	1.000
	Achara	**Adivare**	**Rameshwar-Girye**	**Nadan-Jamsande**	**Katta**	**Ambivali-Kelshi**	**Murud**	**Padel**	**Pawas**	**Vengurle**

Fig. 1: Dendrogram depicting Alphonso samples from 10 locations based on the genetic distance by 16 ISSR primers.

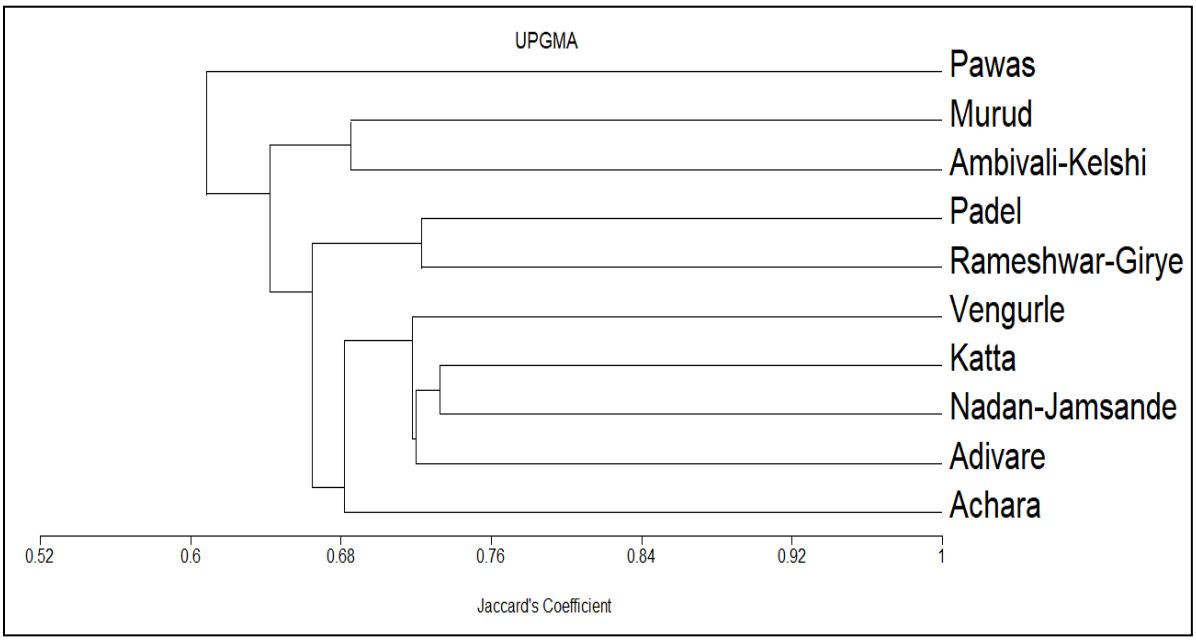

Table 17: ISSR clustering pattern of selected 10 locations of Alphonso

Clusters			No. of locations	Name of Location
I			1	Pawas
II	**IIA**		2	Ambivali-Kelshi and Murud
	IIB	**IIBa**	2	Padel and Rameshwar-Girye
		IIBb	5	Achara, Adivare, Nadan-Jamsande, Katta, Vengurle

4.5.2 ISSR analysis of 50 Alphonso samples within each location

The prime objective of within samples analysis is to assess the genetic variation present within each samples. Sixteen ISSR primers were used to analyze the 5 samples of each location.

4.5.2.1 ISSR analysis within Alphonso samples from Achara

4.5.2.1.1 Per cent polymorphism within Achara

The per cent polymorphism of UBC-884 primer was 100 per cent which is higher than other primers. The amplification range of this primer was found to be within 1.500-1.575 Kb. The lowest polymorphism percentage (19.40 %) was shown by the primer UBC-885 with total number of 31 fragments. The average per cent polymorphism across the 16 primers within five samples of Achara found to be 42.83 per cent (Table 18). The primer UBC-891 produced monomorphic banding pattern. The size of amplified product ranged from 0.320 Kb to 2.832 Kb (UBC-885 and UBC-854).

4.5.2.1.2 Genetic distance within Achara

The genetic distance was computed considering all five samples from the pooled data and the dendrogram was constructed. The distance similarity matrix is based on Jaccard's similarity coefficient is presented in Table 19. The maximum similarity coefficient was observed between sample C1 and C4 i.e. 0.760, while lowest similarity coefficient was observed between sample number C3 and C5 i.e. 0.630. The overall similarity between five samples of Achara was ranged from 0.630 to 0.760 this indicates that there was low variability in Alphonso samples from Achara.

4.5.2.1.3 Cluster analysis within Achara

The dendrogram based on Jaccard's similarity coefficient was constructed using UPGMA after analysis of banding patterns generated by five samples from Achara with 16 ISSR primers. The dendrogram (Fig. 2) separated five samples of Achara into two main clusters, *viz*; I and II. Cluster I contains only one sample C5. Cluster II divided into two sub clusters, *viz*; IIA and IIB. IIA contains sample C3. IIB again sub divided into two clusters IIBa and IIBb. IIBa contains sample C2 while IIBb consists sample C1 and C4 (Table 20).

Table 18: ISSR per cent polymorphism and range of amplification within Achara

Sr. No.	Primer	% Polymorphism	Range of Amplification (Kb)
1.	UBC-811	35.50	0.440-1.994
2.	UBC-812	20.00	0.412-1.109
3.	UBC-815	75.00	0.776-2.410
4.	UBC-817	50.00	0.922-1.108
5.	UBC-818	28.60	0.490-2.224
6.	UBC-834	33.30	0.780-1.458
7.	UBC-853	50.00	0.466-1.116
8.	UBC-854	44.40	0.388-2.832
9.	UBC-857	50.00	0.379-0.510
10.	UBC-876	44.40	0.612-2.182
11.	UBC-881	33.30	0.423-1.998
12.	UBC-884	100.0	1.500-1.575
13.	UBC-885	19.40	0.320-1.335
14.	UBC-886	21.10	0.408-2.086
15.	UBC-889	37.50	0.566-0.978
16.	UBC-891	00.00	0.462-1.474
Average		**42.83**	**0.584-1.649**

Table 19: Genetic similarity coefficient based on ISSRs pooled over the 16 primers within Achara

	C1	C2	C3	C4	C5
C1	1.000				
C2	0.700	1.000			
C3	0.690	0.670	1.000		
C4	0.760	0.740	0.670	1.000	
C5	0.640	0.670	0.630	0.640	1.000
	C1	**C2**	**C3**	**C4**	**C5**

Fig. 2: Dendrogram depicting Achara based on the genetic distance by 16 ISSR primers

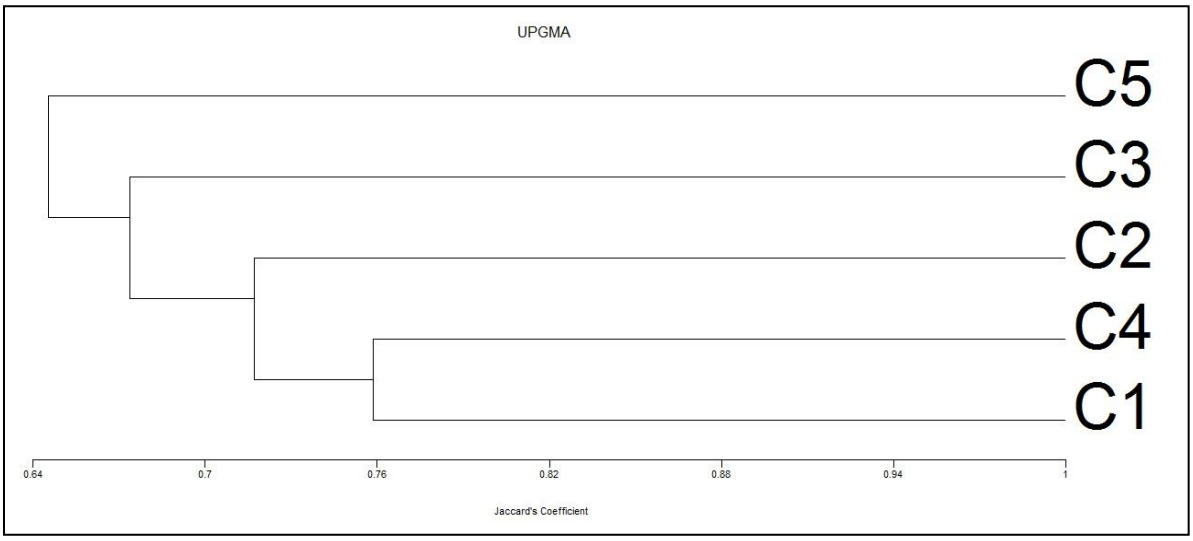

Table 20: ISSR clustering pattern within Achara

Clusters			No. of Samples	Name of Samples
I			1	C5
II	**IIA**		1	C3
	IIB	**IIBa**	1	C2
		IIBb	2	C1, C4

The profile of primer UBC-876 showed that the fragment having size 0.612 Kb was absent in sample C1, C2 and C4. Also sample C3 and A1 from Adivare were not displayed any fragment having size more than 1.900 Kb. Absence of such fragments in particular sample were shown by arrow. Range of amplification of this primer was found within 0.612 Kb to 2.182 Kb (Plate 2).

4.5.2.2 ISSR analysis within Adivare

4.5.2.2.1 Per cent polymorphism within Adivare

The UBC-884 primer revealed 100 per cent polymorphism with minimum number of polymorphic DNA fragments that is 10. The amplification range of this primer was found to be 1.358-1.553 Kb. The lowest polymorphism percentage (10.25%) was shown by the primer UBC-886. The product size ranged from 0.344 Kb to 2.666 Kb (UBC-885 and UBC-854). The average per cent polymorphism across the 16 primers within five samples of Adivare found to be 42.39 per cent. The primer wise per cent polymorphism and range of amplification is given in Table 21.

4.5.2.2.2 Genetic distance within Adivare

From the pooled data, the genetic distance was computed considering five samples of Adivare and the dendrogram was constructed. The overall similarity between five samples of Adivare was ranged from 0.588 to 0.730. The maximum similarity coefficient was observed between sample number A1 and A4 i.e. 0.730, while lowest similarity coefficient was observed between sample number A2 and A5 i.e. 0.588. The distance similarity matrix is based on Jaccard's similarity coefficient is presented in Table 22.

4.5.2.2.3 Cluster analysis within Adivare

The dendrogram based on Jaccard's similarity coefficient was constructed using UPGMA after analysis of banding patterns generated by five samples of Adivare with 16 ISSR primers. The dendrogram (Fig. 3) separated five samples of Adivare into two main clusters, *viz*; I and II. Cluster I contains sample number A2. Cluster II further divided into two sub clusters, *viz*; IIA and IIB where Cluster IA contains sample A3 and A5 while cluster IB consist sample A1 and A4 (Table 23).

Table 21: ISSR per cent polymorphism and range of amplification within Adivare

Sr. No.	Primer	% Polymorphism	Range of Amplification (Kb)
1.	UBC-811	35.50	0.422-1.983
2.	UBC-812	20.00	0.431-1.280
3.	UBC-815	76.20	0.766-2.398
4.	UBC-817	50.00	0.922-1.102
5.	UBC-818	24.20	0.488-2.212
6.	UBC-834	33.30	0.826-1.514
7.	UBC-853	50.00	0.460-1.102
8.	UBC-854	60.00	0.378-2.666
9.	UBC-857	50.00	0.397-0.531
10.	UBC-876	34.20	0.648-2.235
11.	UBC-881	41.20	0.430-2.038
12.	UBC-884	100.0	1.358-1.553
13.	UBC-885	31.00	0.344-1.369
14.	UBC-886	10.25	0.454-2.084
15.	UBC-889	20.00	0.535-0.991
16.	UBC-891	00.00	0.462-1.487
Average		**42.39**	**0.582-1.587**

Table 22: Genetic similarity coefficient based on ISSRs pooled over the 16 primers within Adivare

	A1	A2	A3	A4	A5
A1	1.000				
A2	0.689	1.000			
A3	0.722	0.667	1.000		
A4	0.730	0.674	0.670	1.000	
A5	0.674	0.588	0.707	0.660	1.000
	A1	**A2**	**A3**	**A4**	**A5**

Fig. 3: Dendrogram depicting Adivare based on the genetic distance by 16 ISSR primers.

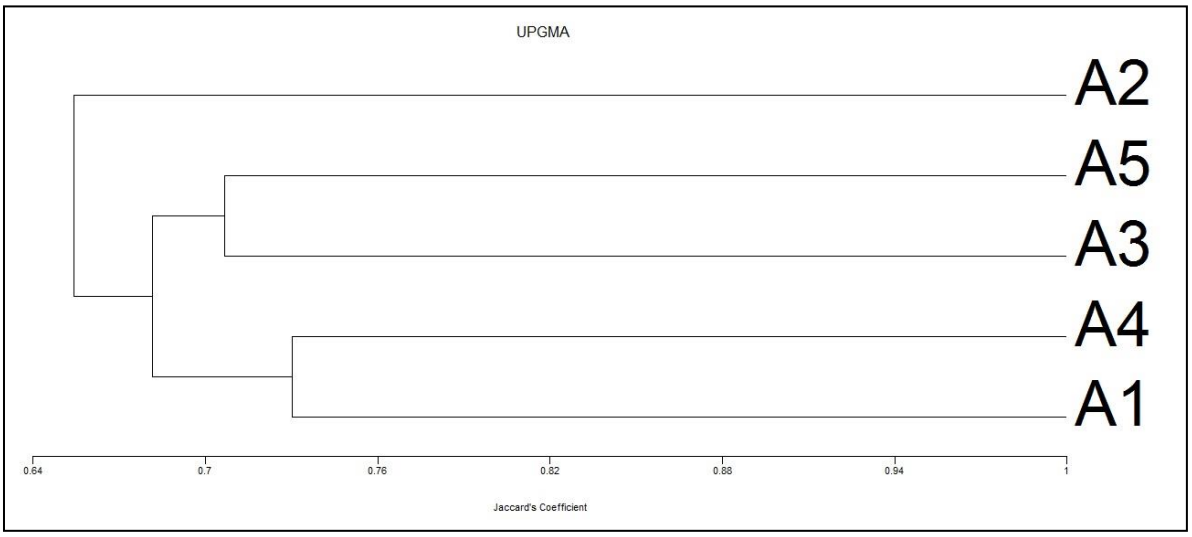

Table 23: ISSR clustering pattern within Adivare

Clusters		No. of Samples	Name of Samples
I		1	A2
II	**IIA**	2	A3, A5
	IIB	2	A1, A4

4.5.2.3 ISSR analysis within Rameshwar-Girye

4.5.2.3.1 Per cent polymorphism within Rameshwar-Girye

The primer UBC-817 and UBC-884 showed 100 per cent polymorphism and produced a total of 10 polymorphic DNA fragments respectively. The amplification of 16 primers ranged from 0.388 Kb to 2.798 Kb (UBC-854). The primer UBC-891 produced monomorphic banding pattern. The primer UBC-886 showed lowest polymorphism percentage (7.14 %) with total 28 DNA fragments. This primer showed absence of two fragments in sample G1 at 1.1 Kb and 2.0 Kb (Plate 3). The primer wise per cent polymorphism and range of amplification is given in Table 24. The average per cent polymorphism across the 16 primers were found to be 42.73 per cent.

4.5.2.3.2 Genetic distance within Rameshwar-Girye

The pair-wise Jaccard's similarity co-efficients for the genetic similarities among the 5 samples are presented in Table 25. The genetic distance was computed considering all the samples from the pooled data and the dendrogram was constructed. Sample G4 and G5 having the lowest similarity coefficient i.e. 0.660. The maximum similarity coefficient was observed between sample G2 and G5 i.e. 0.770. The overall similarity was ranged from 0.660 to 0.770 which indicated the distinctness of these samples from Rameshwar-Girye.

4.5.2.3.3 Cluster analysis within Rameshwar-Girye

The dendrogram based on Jaccard's similarity coefficient was constructed using UPGMA after analysis of banding patterns generated by five samples of Rameshwar-Girye with 16 ISSR primers. The dendrogram (Fig. 4) separated five samples of Rameshwar-Girye into two main clusters, *viz*; I and II. Cluster I further divided into two sub clusters, *viz*; IA and IB where Cluster IA contains sample G3

while cluster IB consist sample G2 and G5. Cluster II contains sample G1and G4 (Table 26).

Table 24: ISSR per cent polymorphism and range of amplification within Rameshwar-Girye

Sr. No.	Primer	% Polymorphism	Range of Amplification (Kb)
1.	UBC-811	19.40	0.443-1.976
2.	UBC-812	20.80	0.402-1.120
3.	UBC-815	73.70	0.744-2.462
4.	UBC-817	100.0	0.880-1.076
5.	UBC-818	37.50	0.480-2.260
6.	UBC-834	33.30	0.816-1.590
7.	UBC-853	50.00	0.476-1.104
8.	UBC-854	60.00	0.388-2.798
9.	UBC-857	50.00	0.395-0.520
10.	UBC-876	18.90	0.634-2.294
11.	UBC-881	23.10	0.458-1.978
12.	UBC-884	100.0	1.500-1.682
13.	UBC-885	31.00	0.391-1.375
14.	UBC-886	7.14	0.430-2.034
15.	UBC-889	20.00	0.544-0.990
16.	UBC-891	00.00	0.462-1.498
Average		**42.73**	**0.590-1.672**

Table 25: Genetic similarity coefficient based on ISSRs pooled over the 16 primers within Rameshwar-Girye

	G1	G2	G3	G4	G5
G1	1.000				
G2	0.760	1.000			
G3	0.740	0.760	1.000		
G4	0.760	0.670	0.710	1.000	
G5	0.710	0.770	0.740	0.660	1.000
	G1	**G2**	**G3**	**G4**	**G5**

Fig. 4: Dendrogram depicting Rameshwar-Girye based on the genetic distance by 16 ISSR primers

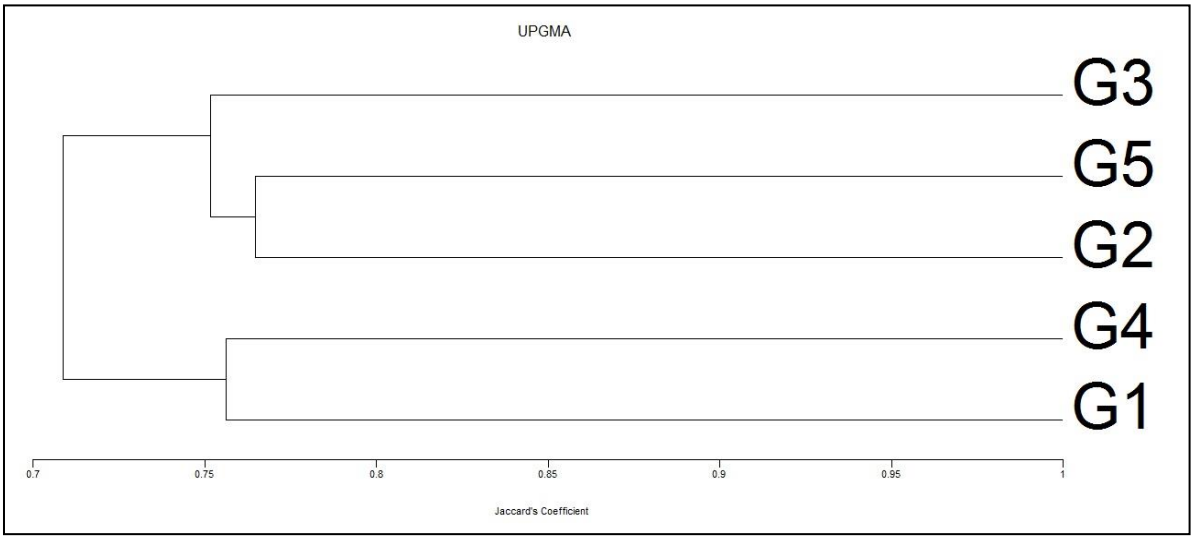

Table 26: ISSR clustering pattern within Rameshwar-Girye

Clusters		No. of Samples	Name of Samples
I	**IA**	1	G3
	IB	2	G2, G5
II		2	G1, G4

4.5.2.4 ISSR analysis within Nadan-Jamsande

4.5.2.4.1 Per cent polymorphism within Nadan-Jamsande

The average per cent polymorphism across the 16 primers within five samples of Nadan-Jamsande was found to be 46.77 per cent. Primer UBC-886 exhibited 12.50 per cent polymorphism. This displayed fragment having size 1.8-1.9 Kb which was observed in sample J3, J4 and J5 but absent in J1 and J2 (Plate 3). UBC-891 produced monomorphic banding pattern. Three primers, UBC-817, UBC-853 and UBC -884 showed the highest polymorphism percentage. The amplified product ranged between 0.377 Kb to 2.842 Kb (UBC-885 and UBC-854). The primer wise per cent polymorphism and range of amplification is given in Table 27.

4.5.2.4.2 Genetic distance within Nadan-Jamsande

The overall similarity was ranged from 0.600 to 0.753 which indicated the distinctness of these samples from Nadan-Jamsande. Sample J1 and J2 having the maximum similarity coefficient i.e. 0.753. The lowest similarity coefficient 0.600 was observed between sample J3 and J4. From the pooled data, the genetic distance was computed considering five samples of Nadan-Jamsande and the dendrogram was constructed. The distance similarity matrix is based on Jaccard's similarity coefficient is presented in Table 28.

4.5.2.4.3 Cluster analysis within Nadan-Jamsande

The dendrogram based on Jaccard's similarity coefficient was constructed using UPGMA after analysis of banding patterns generated by five samples of Nadan-Jamsande with 16 ISSR primers. The dendrogram (Fig. 5) separated five samples of Nadan-Jamsande into two main clusters, *viz*; I and II. Cluster I contains sample J4 and J5. Cluster II further divided into two sub clusters, *viz*; IIA and

IIB. Sub cluster IIA contains sample J3 while cluster IIB consist sample J1 and J2 (Table 29).

Table 27: ISSR per cent polymorphism and range of amplification within Nadan-Jamsande

Sr. No.	Primer	% Polymorphism	Range of Amplification (Kb)
1.	UBC-811	16.70	0.432-1.986
2.	UBC-812	16.70	0.431-1.125
3.	UBC-815	75.00	0.7554-2.489
4.	UBC-817	100.0	0.886-1.078
5.	UBC-818	19.40	0.486-2.223
6.	UBC-834	33.30	0.780-1.510
7.	UBC-853	100.0	0.460-1.102
8.	UBC-854	63.00	0.385-2.842
9.	UBC-857	44.40	0.405-0.518
10.	UBC-876	28.60	0.632-2.240
11.	UBC-881	28.60	0.437-1.985
12.	UBC-884	100.0	1.500-1.617
13.	UBC-885	25.90	0.377-1.323
14.	UBC-886	12.50	0.413-1.972
15.	UBC-889	37.50	0.538-0.989
16.	UBC-891	00.00	0.462-1.487
Average		**46.77**	**0.586-1.655**

Table 28: Genetic similarity coefficient based on ISSRs pooled over the 16 primers within Nadan-Jamsande

	J1	J2	J3	J4	J5
J1	1.000				
J2	0.753	1.000			
J3	0.733	0.741	1.000		
J4	0.663	0.689	0.600	1.000	
J5	0.656	0.663	0.644	0.727	1.000
	J1	**J2**	**J3**	**J4**	**J5**

Fig. 5: Dendrogram depicting Nadan-Jamsande based on the genetic distance by 16 ISSR primers

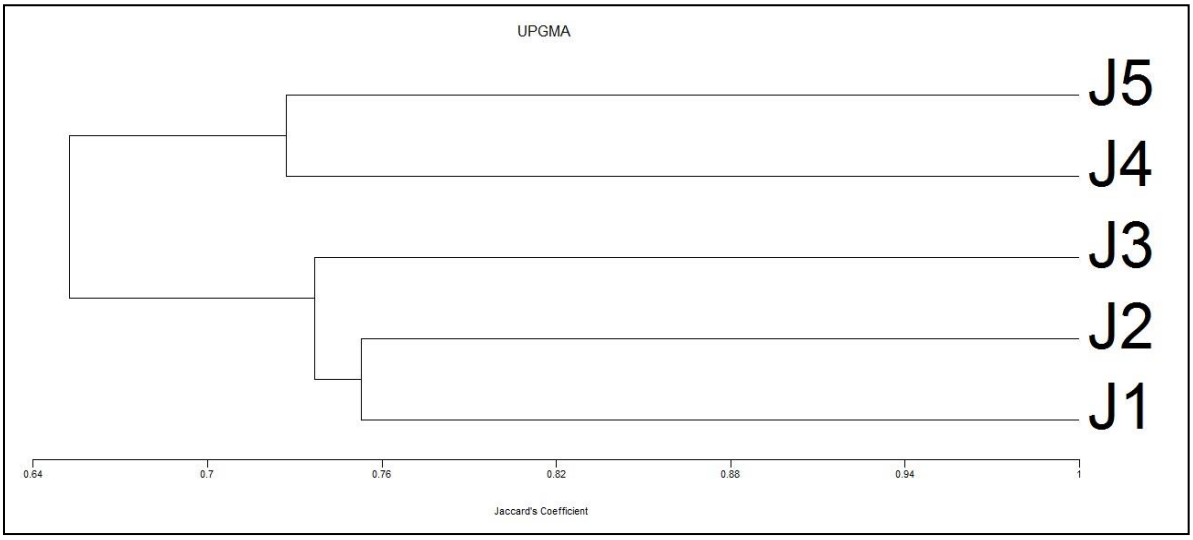

Table 29: ISSR clustering pattern within Nadan-Jamsande

Clusters		No. of Samples	Name of Samples
I		2	J4, J5
II	**IIA**	1	J3
	IIB	2	J1, J2

4.5.2.5 ISSR analysis within Katta

4.5.2.5.1 Per cent polymorphism within Katta.

The primer UBC-817 and UBC-853 both produced a total of 20 DNA fragments and which showed 100 per cent polymorphism across the 5 samples from Katta. The range of amplification of primer UBC-817 was from 0.922-1.074 Kb and primer UBC-853 was from 0.460-1.102 Kb. The overall product size of 16 primers ranged from 0.345 Kb to 2.748 Kb (UBC-885 and UBC-854). In the profile of primer UBC-818 arrow indicated that absence of fragment in sample L1 and L4 (Plate 4). The primer UBC-886 showed the lowest polymorphism percentage (11.80 %). The primer UBC-891 displayed monomorphic banding pattern. The average per cent polymorphism within five samples of Katta was found to be 44.22 per cent. The primer wise per cent polymorphism and range of amplification is given in Table 30.

4.5.2.5.2 Genetic distance within Katta

The genetic distance was computed considering five samples of Katta from the pooled data and the dendrogram was constructed. The pair-wise Jaccard's similarity co-efficients for the genetic similarities among 5 samples are presented in Table 31. The overall similarity between five samples of Katta was ranged from 0.615 (between sample L3 and L4) to 0.793 (between sample L1 and L3) indicated the distinctness of these samples.

4.5.2.5.3 Cluster analysis within Katta

The dendrogram based on Jaccard's similarity coefficient was constructed using UPGMA after analysis of banding patterns generated by five samples of Katta with 16 ISSR primers. The dendrogram (Fig. 6) separated five samples of Katta into two main clusters, *viz*; I and II. Cluster I contains sample L4. Cluster II further

divided into two sub clusters, *viz*; IIA and IIB where Cluster IIA contains sample L2 and L5 while cluster IB consist sample L1 and L3. (Table 32).

Table 30: ISSR per cent polymorphism and range of amplification within Katta

Sr. No.	Primer	% Polymorphism	Range of Amplification (Kb)
1.	UBC-811	50.00	0.438-1.992
2.	UBC-812	20.00	0.434-1.011
3.	UBC-815	50.00	0.736-2.438
4.	UBC-817	100.0	0.922-1.074
5.	UBC-818	19.40	0.484-2.188
6.	UBC-834	33.30	0.794-1.522
7.	UBC-853	100.0	0.460-1.102
8.	UBC-854	60.00	0.376-2.748
9.	UBC-857	50.00	0.380-0.514
10.	UBC-876	42.90	0.646-2.298
11.	UBC-881	39.40	0.472-2.005
12.	UBC-884	50.00	1.453-1.742
13.	UBC-885	16.70	0.345-1.353
14.	UBC-886	11.80	0.466-2.138
15.	UBC-889	20.00	0.558-0.986
16.	UBC-891	00.00	0.460-1.478
Average		**44.22**	**0.589-1.661**

Table 31: Genetic similarity coefficient based on ISSRs pooled over the 16 primers within Katta

	L1	L2	L3	L4	L5
L1	1.000				
L2	0.750	1.000			
L3	0.793	0.701	1.000		
L4	0.718	0.709	0.615	1.000	
L5	0.753	0.744	0.648	0.674	1.000
	L1	**L2**	**L3**	**L4**	**L5**

Fig. 6: Dendrogram depicting Katta based on the genetic distance by 16 ISSR primers

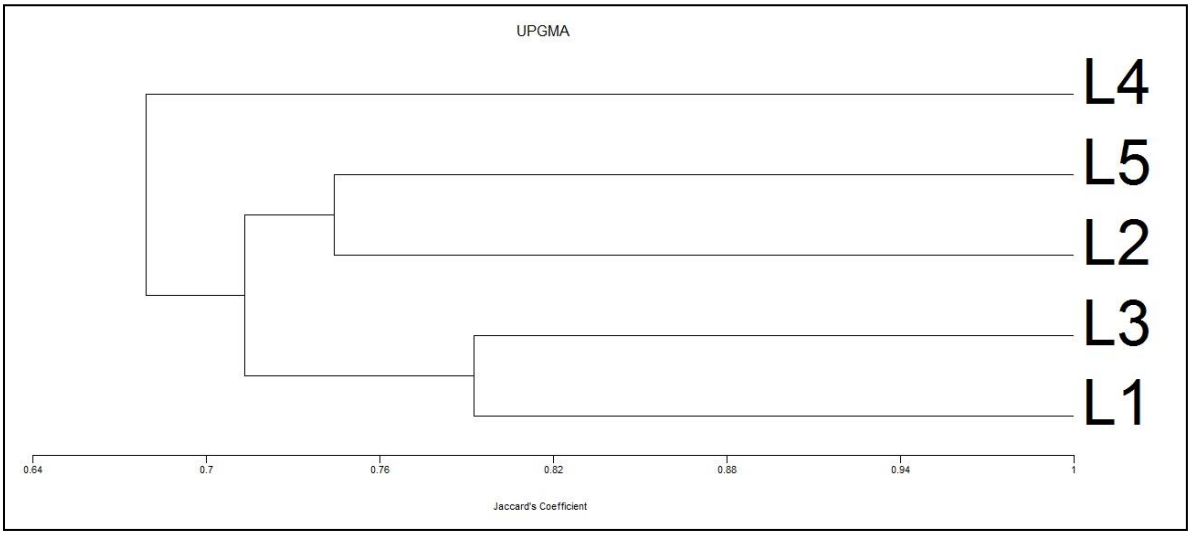

Table 32: ISSR clustering pattern within Katta

Clusters		No. of Samples	Name of Samples
I		1	L4
II	**IIA**	2	L2, L5
	IIB	2	L1, L3

4.5.2.6 ISSR marker analysis within Ambivali-Kelshi

4.5.2.6.1 Per cent polymorphism within Ambivali-Kelshi

The average per cent polymorphism within five samples of Ambivali-Kelshi was found to be 44.58 per cent. Three primers showed the 100 per cent polymorphism *viz.*, UBC-817, UBC-853 and UBC-884. The lowest polymorphism percentage (3.23 %) was shown by the primer UBC-886 with total number of 31 amplified fragments. In the profile of primer UBC-885 arrow indicated that absence of fragment in sample K1 and K2 (Plate 4). The all amplified product size ranged from 0.374 Kb to 2.661 Kb (UBC-854). Monomorphic banding pattern was produced by the primer UBC-891. The primer wise per cent polymorphism and range of amplification is given in Table 33.

4.5.2.6.2 Genetic distance within Ambivali-Kelshi

The maximum similarity coefficient was observed between sample K1 and K5 i.e. 0.790, while lowest similarity coefficient was observed between sample number K1 and K2 i.e. 0.593. The overall similarity between five samples of Ambivali-Kelshi was ranged from 0.593 to 0.790 indicated distinctness of these samples. The genetic distance was computed considering all five samples from the pooled data and the dendrogram was constructed. The distance similarity matrix is based on Jaccard's similarity coefficient is presented in Table 34.

4.5.2.6.3 Cluster analysis within Ambivali-Kelshi

The dendrogram based on Jaccard's similarity coefficient was constructed using UPGMA after analysis of banding patterns generated by five samples of Ambivali-Kelshi with 16 ISSR primers. The dendrogram (Fig. 7) separated five samples of Ambivali-Kelshi into two main clusters, *viz*; I and II. Cluster I contains only one sample K2. Cluster II divided into two sub

clusters, *viz*; IIA and IIB. IIA contains sample K4. IIB again sub divided into two clusters IIBa and IIBb. IIBa contains sample K3 while IIBb consists sample K1 and K5 (Table 35).

Table 33: ISSR per cent polymorphism and range of amplification within Ambivali-Kelshi

Sr. No.	Primer	% Polymorphism	Range of Amplification (Kb)
1.	UBC-811	14.30	0.443-1.981
2.	UBC-812	37.50	0.436-1.112
3.	UBC-815	75.00	0.755-2.416
4.	UBC-817	100.0	0.896-1.112
5.	UBC-818	16.70	0.478-2.257
6.	UBC-834	33.30	0.812-1.598
7.	UBC-853	100.0	0.480-1.132
8.	UBC-854	60.00	0.374-2.661
9.	UBC-857	50.00	0.397-0.512
10.	UBC-876	24.20	0.639-2.343
11.	UBC-881	16.00	0.471-1.987
12.	UBC-884	100.0	1.500-1.718
13.	UBC-885	20.00	0.377-1.354
14.	UBC-886	3.23	0.438-2.068
15.	UBC-889	20.00	0.536-0.998
16.	UBC-891	00.00	0.457-1.500
	Average	**44.58**	**0.593-1.671**

Table 34: Genetic similarity coefficient based on ISSRs pooled over the 16 primers within Ambivali-Kelshi

	K1	K2	K3	K4	K5
K1	1.000				
K2	0.593	1.000			
K3	0.753	0.644	1.000		
K4	0.759	0.615	0.714	1.000	
K5	0.790	0.718	0.765	0.729	1.000
	K1	**K2**	**K3**	**K4**	**K5**

Fig. 7: Dendrogram depicting Ambivali-Kelshi based on the genetic distance by 16 ISSR primers

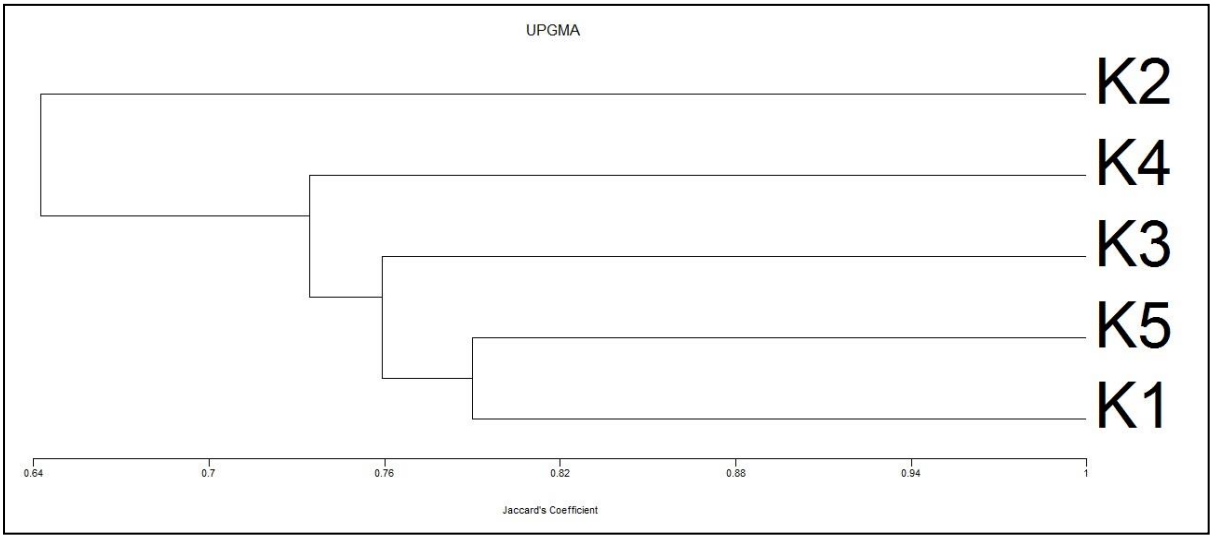

Table 35: ISSR clustering pattern within Ambivali-Kelshi

Clusters			No. of Samples	Name of Samples
I			1	K2
II	**IIA**		1	K4
	IIB	**IIBa**	1	K3
		IIBb	2	K1, K5

4.5.2.7 ISSR analysis within Murud

4.5.2.7.1 Per cent polymorphism within Murud

The UBC-817 and UBC-853 primers both revealed 100 per cent polymorphism with minimum number of polymorphic DNA fragments that is 10. The lowest polymorphism percentage (14.30 %) was shown by the primer UBC-886 having amplified product size between 0.449-2.144 Kb. Sample M2 displayed unique fragment in the profile of primer UBC-815 (Plate 5). The amplification product of all 16 primers size ranged from 0.328 Kb to 2.780 Kb (UBC-885 and UBC-854). The average per cent polymorphism within five samples of Murud was found to be 46.29 per cent. Monomorphic banding pattern was produced by the primers UBC-891 and UBC-834. The primer wise per cent polymorphism and range of amplification is given in Table 36.

4.5.2.7.2 Genetic distance within Murud

From the pooled data, the genetic distance was computed considering five samples of Murud and the dendrogram was constructed. The overall similarity between five samples of Murud was ranged from 0.602 to 0.776. The maximum similarity coefficient was observed between sample number M1 and M3 i.e. 0.776, while lowest similarity coefficient was observed between sample number M2 and M3 i.e. 0.602. The distance similarity matrix is based on Jaccard's similarity coefficient is presented in Table 37.

4.5.2.7.3 Cluster analysis within Murud

The dendrogram based on Jaccard's similarity coefficient was constructed using UPGMA after analysis of banding patterns generated by five samples of Murud with 16 ISSR primers. The dendrogram (Fig. 8) separated five samples of

Murud into two main clusters, *viz*; I and II. Cluster I contains sample M2. Cluster II further divided into two sub clusters, *viz*; IIA and IIB where Cluster IIA contains sample M4 and M5 while cluster IIB consist sample M1 and M3 (Table 38).

Table 36: ISSR per cent polymorphism and range of amplification within Murud

Sr. No.	Primer	% Polymorphism	Range of Amplification (Kb)
1.	UBC-811	35.50	0.436-1.948
2.	UBC-812	20.00	0.428-1.109
3.	UBC-815	21.34	0.746-1.698
4.	UBC-817	100.0	0.899-1.068
5.	UBC-818	24.20	0.488-2.198
6.	UBC-834	00.00	0.808-1.500
7.	UBC-853	100.0	0.452-1.124
8.	UBC-854	60.00	0.398-2.780
9.	UBC-857	50.00	0.390-0.502
10.	UBC-876	35.90	0.639-2.301
11.	UBC-881	35.50	0.437-2.038
12.	UBC-884	50.00	1.500-1.701
13.	UBC-885	31.00	0.328-1.338
14.	UBC-886	14.30	0.449-2.144
15.	UBC-889	16.70	0.460-0.990
16.	UBC-891	00.00	0.444-1.495
Average		**46.29**	**0.581-1.663**

Table 37: Genetic similarity coefficient based on ISSRs pooled over the 16 primers within Murud

	M1	M2	M3	M4	M5
M1	1.000				
M2	0.644	1.000			
M3	0.776	0.602	1.000		
M4	0.708	0.685	0.645	1.000	
M5	0.708	0.648	0.700	0.711	1.000
	M1	**M2**	**M3**	**M4**	**M5**

Fig. 8: Dendrogram depicting Murud based on the genetic distance by 16 ISSR primers

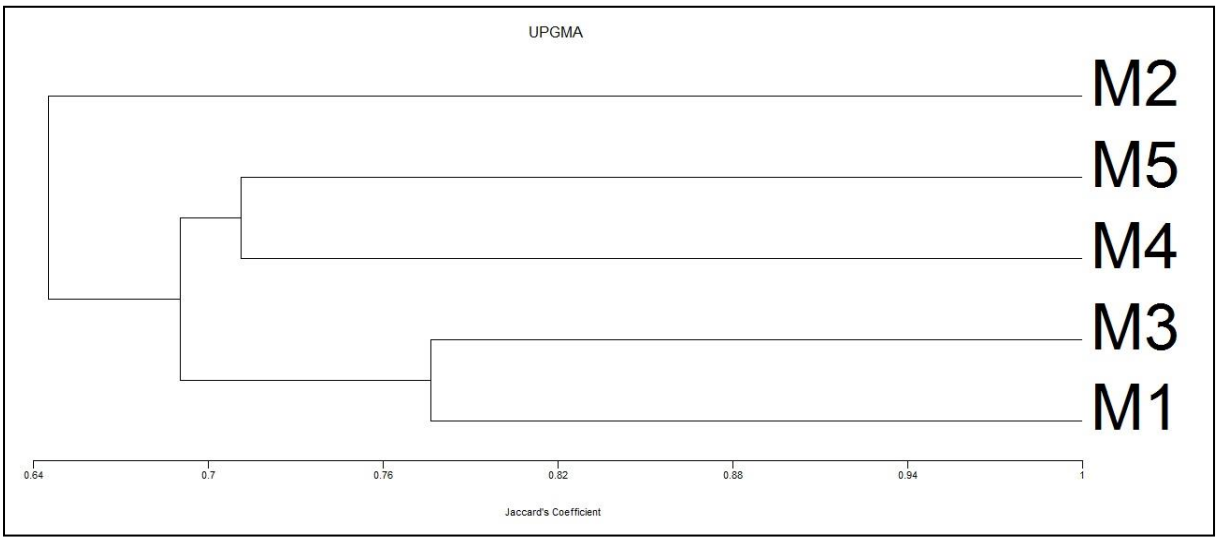

Table 38: ISSR clustering pattern within Murud

Clusters		No. of Samples	Name of Samples
I		1	M2
II	**IIA**	2	M4, M5
	IIB	2	M1, M3

4.5.2.8 ISSR analysis within Padel

4.5.2.8.1 Per cent polymorphism within Padel

The highest per cent polymorphism was shown by primer UBC-815 with total number of 18 amplified fragments i.e. 72.20 %. The amplification of 16 primers ranged from 0.345-2.694 Kb (UBC-885 and UBC-854). Unique fragments were observed in sample D1 and D4 which is revealed by the primer UBC-817 (Plate 5). The primer UBC-889 showed lowest polymorphism percentage (3.85 %) with total 26 DNA fragments. The primer UBC-891 produced monomorphic banding pattern. The primer wise per cent polymorphism and range of amplification is given in Table 39. The average per cent polymorphism across the 16 primers found to be 30.45 per cent.

4.5.2.8.2 Genetic distance within Padel

The pair-wise Jaccard's similarity coefficients for the genetic similarities among the 5 samples are presented in Table 40. The genetic distance was computed considering all the samples from the pooled data and the dendrogram was constructed. Sample D2 and D3 having the lowest similarity coefficient i.e. 0.656. The maximum similarity coefficient was observed between sample D1 and D4 i.e. 0.786. The overall similarity was ranged from 0.656 to 0.786 which indicated the distinctness of these samples from Padel.

4.5.2.8.3 Cluster analysis within Padel

The dendrogram based on Jaccard's similarity coefficient was constructed using UPGMA after analysis of banding patterns generated by five samples of Padel with 16 ISSR primers. The dendrogram (Fig. 9) separated five samples of Padel into two main clusters, *viz*; I and II. Cluster I contains sample D2 and D5. Cluster II further divided into two sub

clusters, *viz*; IIA and IIB where Cluster IIA contains sample D3 while cluster IIB consist sample D1 and D4 (Table 41).

Table 39: ISSR per cent polymorphism and range of amplification within Padel.

Sr. No.	Primer	% Polymorphism	Range of Amplification (Kb)
1.	UBC-811	19.40	0.443-1.988
2.	UBC-812	20.00	0.434-1.110
3.	UBC-815	72.20	0.758-2.423
4.	UBC-817	50.00	0.920-1.096
5.	UBC-818	23.10	0.470-2.190
6.	UBC-834	9.09	0.831-1.577
7.	UBC-853	28.60	0.462-1.108
8.	UBC-854	30.80	0.368-2.694
9.	UBC-857	37.50	0.399-0.515
10.	UBC-876	30.60	0.636-2.267
11.	UBC-881	33.30	0.437-2.072
12.	UBC-884	54.50	1.526-1.733
13.	UBC-885	37.50	0.345-1.338
14.	UBC-886	6.45	0.444-2.154
15.	UBC-889	3.85	0.536-0.986
16.	UBC-891	00.00	0.458-1.459
Average		**30.45**	**0.591-1.669**

Table 40: Genetic similarity coefficient based on ISSRs pooled over the 16 primers within Padel

	D1	D2	D3	D4	D5
D1	1.000				
D2	0.674	1.000			
D3	0.783	0.656	1.000		
D4	0.786	0.677	0.744	1.000	
D5	0.736	0.744	0.756	0.700	1.000
	D1	**D2**	**D3**	**D4**	**D5**

Fig. 9: Dendrogram depicting Padel based on the genetic distance by 16 ISSR primers.

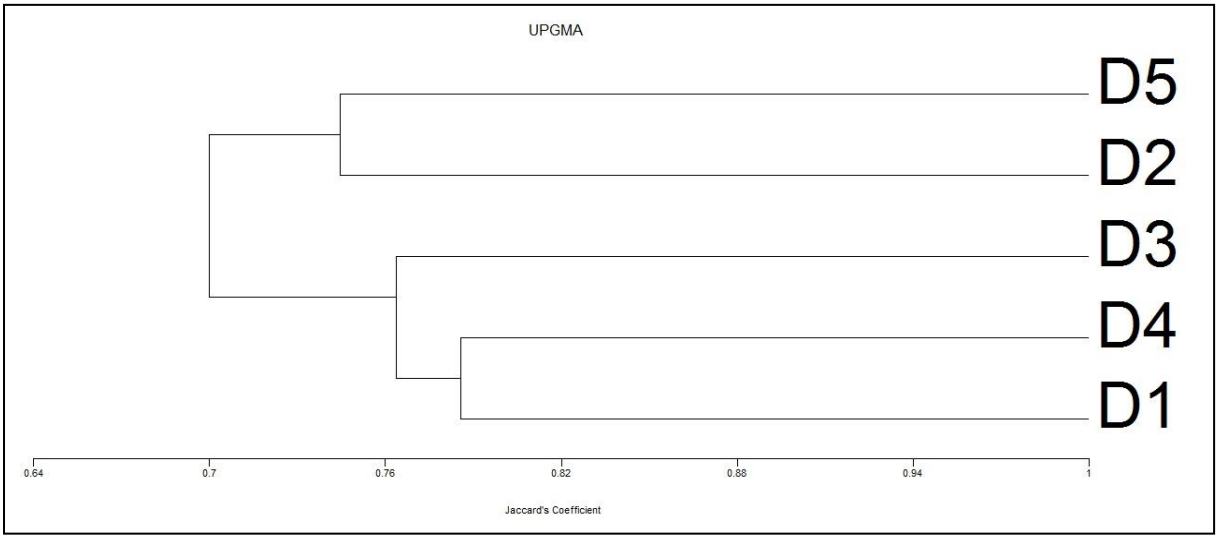

Table 41: ISSR clustering pattern within Padel

Clusters		No. of Samples	Name of Samples
I		2	D2, D5
II	**IIA**	1	D3
	IIB	2	D1, D4

4.5.2.9 ISSR analysis within Pawas

4.5.2.9.1 Per cent polymorphism within Pawas

The primer UBC-817 and UBC-884 both produced a total of 20 DNA fragments and which showed 100 per cent polymorphism across the 5 samples from Pawas. The range of amplification of primer UBC-817 was from 0.924-1.024 Kb and primer UBC-884 was from 1.500-1.729 Kb. The overall product size of 16 primers ranged from 0.372-2.891 Kb (UBC-885 and UBC-854). The primer UBC-811 showed the lowest polymorphism percentage (14.30 %). The average per cent polymorphism within five samples of Pawas was found to be 47.05 per cent. The primer UBC-886 and UBC-891 both produced monomorphic banding pattern (Plate 6). The primer wise per cent polymorphism and range of amplification is given in Table 42.

4.5.2.9.2 Genetic distance within the samples from Pawas

The genetic distance was computed considering five samples of Pawas from the pooled data and the dendrogram was constructed. The pair-wise Jaccard's similarity co-efficients for the genetic similarities among 5 samples are presented in Table 43. The overall similarity between five samples of Pawas was ranged from 0.617 (between sample P4 and P5) to 0.774 (between sample P1 and P5) indicated the distinctness of these samples.

4.5.2.9.3 Cluster analysis within Pawas

The dendrogram based on Jaccard's similarity coefficient was constructed using UPGMA after analysis of banding patterns generated by five samples of Pawas with 16 ISSR primers. The dendrogram (Fig. 10) separated five samples of Pawas into two main clusters, *viz*; I and II. Cluster I further divided into two sub clusters, *viz*; IA and IB. Sub cluster IA

contains sample P3 while cluster IB consist sample P2 and P4. Cluster II contains sample P1 and P5 (Table 44).

Table 42: ISSR per cent polymorphism and range of amplification within Pawas.

Sr. No.	Primer	% Polymorphism	Range of Amplification (Kb)
1.	UBC-811	14.30	0.443-1.983
2.	UBC-812	37.50	0.434-1.108
3.	UBC-815	75.00	0.756-2.422
4.	UBC-817	100.0	0.924-1.024
5.	UBC-818	16.70	0.484-2.066
6.	UBC-834	33.30	0.837-1.546
7.	UBC-853	50.00	0.440-1.124
8.	UBC-854	56.50	0.378-2.891
9.	UBC-857	50.00	0.379-0.517
10.	UBC-876	46.20	0.622-2.278
11.	UBC-881	25.90	0.475-1.955
12.	UBC-884	100.0	1.500-1.729
13.	UBC-885	33.30	0.372-1.348
14.	UBC-886	00.00	0.434-2.066
15.	UBC-889	20.00	0.587-0.980
16.	UBC-891	00.00	0.462-1.487
Average		**47.05**	**0.595-1.657**

Table 43: Genetic similarity coefficient based on ISSRs pooled over the 16 primers within Pawas.

	P1	P2	P3	P4	P5
P1	1.000				
P2	0.750	1.000			
P3	0.718	0.747	1.000		
P4	0.716	0.765	0.713	1.000	
P5	0.774	0.626	0.652	0.617	1.000
	P1	**P2**	**P3**	**P4**	**P5**

Fig. 10: Dendrogram depicting Pawas based on the genetic distance by 16 ISSR primers.

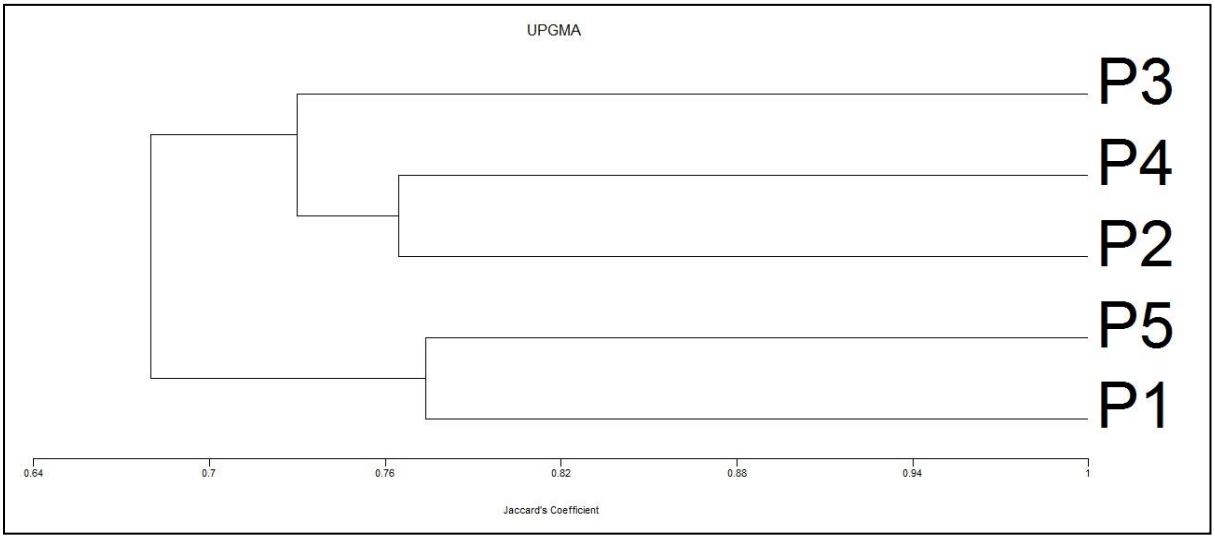

Table 44: ISSR clustering pattern within Pawas

Clusters		No. of Samples	Name of Samples
I	**IA**	1	P3
	IB	2	P2, P4
II		2	P1, P5

4.5.2.10 ISSR analysis within Vengurle

4.5.2.10.1 Per cent polymorphism within Vengurle

The UBC-857 primer revealed 100 per cent polymorphism with minimum number of polymorphic DNA fragments that is 10. The amplification range of this primer was found to be 0.375-0.510 Kb. The lowest polymorphism percentage (3.23 %) was shown by the primer UBC-886. The product size ranged from 0.351 Kb to 2.853 Kb (UBC-885 and UBC-854). The average per cent polymorphism across the 16 primers within five samples of Vengurle was found to be 42.67 per cent. The primer UBC-891 produced monomorphic banding pattern (Plate 6). The primer wise per cent polymorphism and range of amplification is given in Table 45.

4.5.2.10.2 Genetic distance within Vengurle

Sample V3 and V4 having the maximum similarity coefficient i.e. 0.855. The lowest similarity coefficient 0.648 was observed between sample V2 and V5. The overall similarity was ranged from 0.648 to 0.855 which indicated the distinctness of these samples from Vengurle. The genetic distance was computed considering five samples of Vengurle from the pooled data and the dendrogram was constructed. The distance similarity matrix is based on Jaccard's similarity coefficient is presented in Table 46.

4.5.2.10.3 Cluster analysis within Vengurle

The dendrogram based on Jaccard's similarity coefficient was constructed using UPGMA after analysis of banding patterns generated by five samples of Vengurle with 16 ISSR primers. The dendrogram (Fig. 11) separated five samples of Vengurle into two main clusters, *viz*; I and II. Cluster I contains sample V2. Cluster II further divided into two sub clusters, *viz*;

IIA and IIB where Cluster IIA contains sample V3 and V4 while cluster IIB consist sample V1 and V5 (Table 47).

Table 45: ISSR per cent polymorphism and range of amplification within Vengurle.

Sr. No.	Primer	% Polymorphism	Range of Amplification (Kb)
1.	UBC-811	33.30	0.441-1.976
2.	UBC-812	20.00	0.440-1.132
3.	UBC-815	76.20	0.764-2.452
4.	UBC-817	50.00	0.918-1.132
5.	UBC-818	21.90	0.486-2.243
6.	UBC-834	47.40	0.815-1.486
7.	UBC-853	50.00	0.460-1.102
8.	UBC-854	60.00	0.394-2.853
9.	UBC-857	100.0	0.375-0.510
10.	UBC-876	48.70	0.646-2.334
11.	UBC-881	39.40	0.442-2.060
12.	UBC-884	50.00	1.484-1.737
13.	UBC-885	20.00	0.351-1.356
14.	UBC-886	3.23	0.474-2.140
15.	UBC-889	20.00	0.557-0.993
16.	UBC-891	00.00	0.464-1.487
Average		**42.67**	**0.594-1.687**

Table 46: Genetic similarity coefficient based on ISSRs pooled over the 16 primers within Vengurle.

	V1	V2	V3	V4	V5

V1	1.000				
V2	0.659	1.000			
V3	0.705	0.716	1.000		
V4	0.674	0.649	0.855	1.000	
V5	0.753	0.648	0.693	0.700	1.000
	V1	**V2**	**V3**	**V4**	**V5**

Fig. 11: Dendrogram depicting Vengurle based on the genetic distance by 16 ISSR primers.

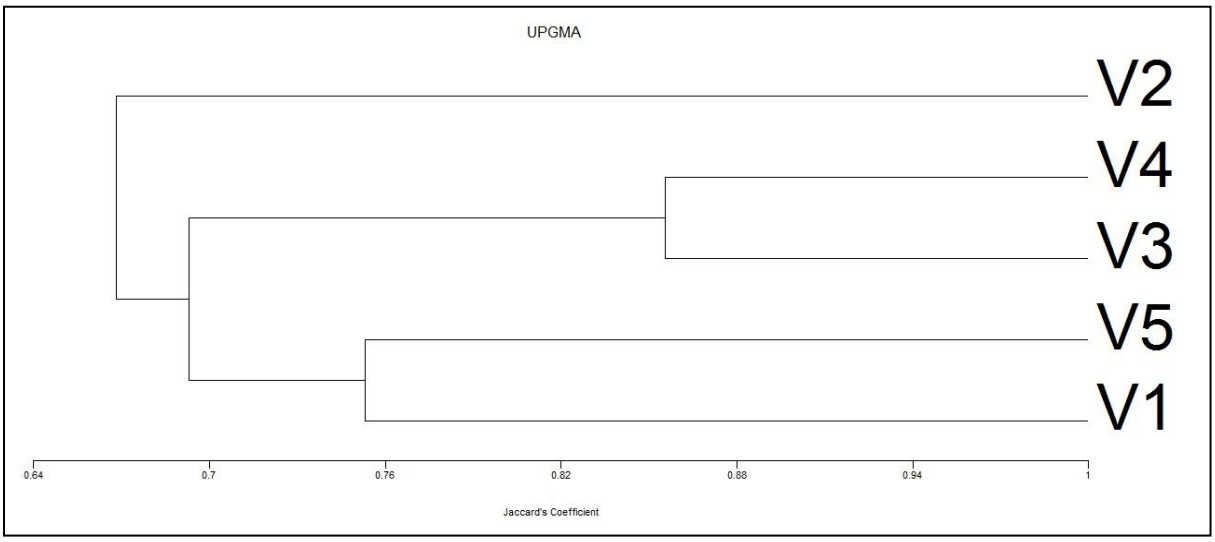

Table 47: ISSR clustering pattern within Vengurle

Clusters		**No. of Samples**	**Name of Samples**
I		1	V2
II	**IIA**	2	V3, V4
	IIB	2	V1, V5

4.6 Polymorphic information content

ISSR markers were highly informative and polymorphic as evident from its PIC value. The PIC value of each marker, which can be evaluated on the basis of its alleles, varied greatly for all tested ISSR loci. It was calculated to find out the efficiency of

primers in distinguishing individual Alphonso sample. The average PIC values of 16 ISSR markers are given in Table 48. The ISSR profile generated by each primer was analyzed using standard DNA marker (100 bp) and compared with their respective banding pattern.

The highest PIC value shown by the primer UBC-876 was 0.884, followed by the primer UBC-886. The lowest PIC value shown by the primer UBC-891 was 0.00. Average polymorphic information content was 0.733 among the all 50 samples of Alphonso. It indicates that ISSR markers have a great potential to establish molecular signature of Alphonso from different locations.

Table 48: Average PIC values 16 ISSR markers

Sr. No.	Primer	PIC value	Sr. No.	Primer	PIC value
1.	UBC-811	0.825	9.	UBC-857	0.601
2.	UBC-812	0.824	10.	UBC-876	0.884
3.	UBC-815	0.849	11.	UBC-881	0.861
4.	UBC-817	0.632	12.	UBC-884	0.678
5.	UBC-818	0.864	13.	UBC-885	0.846
6.	UBC-834	0.698	14.	UBC-886	0.867
7.	UBC-853	0.629	15.	UBC-889	0.812
8.	UBC-854	0.862	16.	UBC-891	0.00
Average PIC Value			**0.733**		

CHAPTER V
DISCUSSION

Mango is rightly known as “King of fruits”, on account of its extensive cultivation and most appealing taste, among different tropical fruits it is widely accepted by consumers. Although the crop is characterized by having enormous cultivars, only a group of three to four cultivars occupy more than 60 per cent of mango area in our country. ‘Alphonso’ is one such cultivar, preferred by domestic as well as foreign fruit markets. The cultivar is highly popular in Konkan region of Maharashtra and Karnataka regions. But due to the cross-pollination genetic diversity is also observed within Alphonso variety. Probably, genetic variation found within variety of Alphonso is not good for its economic value (Rosetti *et al.*, 2004; Prasanth *et al.*, 2007).

Establishment of molecular profile of any plant is useful for detection of genetic variation present among varieties and within variety. Also knowledge of variability present in the population is essential to crop improvement. Genetic variability is normally assessed by common morphological traits. However, such traits are affected by effects of environment, development stage of the plant and the type of plant material. It also requires several replications to establish the genotypic contributions. Hence, there is a need to go in for a highly reliable and precise method for assessment of genetic diversity with molecular markers overcome this problem.

Molecular markers are known for their rapid and widely used to study the genetic diversity, identify redundancies in germplasm collections, test accession stability and integrity, and

resolve taxonomic relationships. The use of molecular markers allow the direct assessment of genotypic variation at the DNA level. Marker analysis helps to understand the genetic makeup of the accessions and makes it possible to analyze the global organization of genetic variability within a species. They are neutral, not related to age and tissue type, and not influenced by the environmental conditions, have feasibility and lower costs, and are more informative than morphological markers. Inter simple Sequence Repeats (ISSR) have been proven useful for detecting genetic polymorphism among accessions by generating a large number of markers that target multiple microsatellite loci distributed across the genome. ISSR are often used because of their capability of reproducibility, with no gene sequence information, and prior genetic studies are required for the analysis. ISSR markers have been reported to undertake the assessment of genetic diversity of mango cultivars (Ariffin *et al.*, 2015).

On this account, present investigation was carried out with an objective to establish the molecular profile of mango (*Mangifera indica* L.) Cv. Alphonso from different locations of south konkan by using molecular markers. The results obtained on polymorphic amplification products, genetic distance, discrimination power of the primers and grouping of selected locations are discussed in this chapter.

5.1 DNA isolation

The isolation of high-quality DNA is important in any molecular biology work because contaminants such as proteins, polyphenols, and polysaccharides which may interfere with enzymes such as restriction enzymes (in blotting techniques) and *Taq* polymerase (in polymerase chain reaction [PCR]).

Moreover, the contaminating RNA that precipitates along with DNA causes many problems including suppression of PCR amplification (Padmalatha and Prasad, 2006). Thus, it is important to choose the appropriate part of the plant to use as source of DNA and to establish an optimal extraction protocol to yield high quality DNA (Angeles *et al.*, 2005).

For the present experimental study, tender leaf samples were collected from the Alphonso variety. A total of 50 samples were selected for extraction of genomic DNA from selected 10 locations (5 from each location). Angeles *et al.,* (2005) used the similar plant part for DNA isolation. During the course of investigation, the DNA was isolated from the Alphonso leaf by rapid method following the protocol of Edwards *et al.,* (1991) with slight modifications of buffer composition and concentration. Some other researcher's protocol also followed for the isolation of high-quality of DNA (Sahu *et al.*, 2012; Huang *et al.*, 2013). In the present study various concentrations of glucose, PVP, SDS, were tried. Among them, a combination of 0.900 g glucose, 0.100 g PVP, 0.040g sodium bisulphite, 0.050 g lauryl sulphate and 500 µl sarcosyl in 10 ml of extraction buffer (EB) yielded an appropriate quantity of DNA without any phenolic contamination.

Reduction of sample size in the first requirement of DNA extraction procedure which decreases the mucilage formation ultimately reduces the impurities. Incubation at 65°C for 45 min. provided elimination of major left over impurities after crushing, followed by treatments of Chloroform: Isoamyl Alcohol (CIA-24:1). Treatment of CIA helps to separate proteins and polysaccharides from DNA. Huang *et al.*, (2013) given the similar

treatment. Isopropanol alcohol treatments allowed maximum precipitation of DNA for overnight at -20°C. A prolonged and chilled overnight incubation treatment provides more precipitation of DNA (Sahu *et al.*, 2012). The last treatment of 70 per cent chilled ethanol is given to remove excess salts from pelleted DNA (Sahu *et al.*, 2012). The overall modifications in the concentration of different chemicals and steps of extraction protocol of rapid method gave good quality and quantity of DNA which used in further molecular analysis.

5.2 Dilution of crude extract of DNA

PCR requires very appropriate and little quantity of DNA (10-30 ng). If concentration is above or below the optimum there will not be proper amplification of the DNA (Preetha and Subramanian, 2007). The quantity of DNA which is able to amplify was obtained by diluting the crude extract of DNA in double distilled water. Most of the samples diluted up to the ratio 1: 100 (where 1 ml of crude DNA and 100 ml of DDW).

5.3 PCR master mix

PCR Master Mix is a ready-to-use solution containing all the necessary reaction components at optimal concentrations for efficient PCR amplification of DNA templates. These components are discussed below:

5.3.1 Template DNA

The clear banding pattern was observed with 1 µl of diluted crude extract. This quantity given 40 ng of template DNA for reaction Padmalatha and Prasad (2006) observed the clarity of bands with 1 µl of template DNA, and there was the presence of smearing at higher concentrations of the DNA template, which

affected the repeatability; there was an absence of amplification with lower concentration.

5.3.2 Magnesium chloride ($MgCl_2$)

The magnesium ion (Mg^{++}) is required as cofactor for *Taq* DNA polymerase enzyme. The magnesium concentration affect primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer-dimer artifacts, and enzyme activity and fidelity (Kumari *et al.*, 2015). In this study 0.5 µl of 25 mM $MgCl_2$ showed clear amplification. The lower or higher concentration of $MgCl_2$ affect the non-specificity and yield of the PCR product (Padmalatha and Prasad, 2006).

5.3.3 Deoxynucleotide triphosphates (dNTPs)

DNTPs are the nucleotide bases added to the growing DNA strand by the DNA polymerase. It is very important to have equal concentrations of each dNTP (dATP, dCTP, dGTP, dTTP), as inaccuracy in the concentration of even a single dNTP dramatically increases the misincorporation level. Present investigation was done with 1.0 µl of 10 mM dNTPs provided clear banding pattern. Kumari *et al.*, (2015) observed that high concentrations of dNTP reducesd free Mg, interfering with the enzyme. Low dNTP concentrations minimize mispriming at nontarget sites and reduce the likelihood of extending misincorporated nucleotides.

5.3.4 Primers

PCR primers are short fragments of single stranded DNA (15-30 nucleotides in length) that are complementary to DNA sequences that flank the target region of interest. The purpose of PCR primers is to provide a free 3'-OH group to which the DNA

polymerase can add dNTPs. Primers containing sequences complementary to the target region along with a DNA polymerase are key components to enable selective and repeated amplification (Azhar *et al.*, 2013). Primer concentrations between 0.1 and 1 µM are generally optimal. Lower and higher concentrations lead to absence of amplification and primer dimer formation, respectively (Padmalatha and Prasad, 2006). Kumari *et al.*, (2015) observed that high concentrations of ISSR primer may raise the probability of generating a template independent artifact, termed a primer-dimer, and could promote mispriming and accumulation of non-specific product.

5.3.5 *Taq* DNA polymerase

This DNA polymerase is isolated from the bacterium *Thermus aquaticus*, which lives in hot environments and requires biomolecules that are heat stable. Therefore, *Taq* DNA polymerase can efficiently synthesize DNA under the heat intensive conditions of the PCR reaction. However, the *Taq* polymerase employed exhibited clear bands with 0.5 µl *Taq* polymerase (3 U/µl). Lower concentration of *Taq* polymerase than optimal did not show proper amplification while high concentration showed decreased specificity (Padmalatha and Prasad, 2006).

PCR master mix was standardized for ISSR marker for each PCR component and the optimum concentration of each component in master mix which gave better amplification was used for further work.

5.4 Thermo profile

A good annealing temperature was based on distinctive band(s) appeared at specific temperature (Azhar *et al.*, 2013). The higher temperature amplification of large fragments was favored while lower temperatures favored short fragment amplification (Mohamad *et al.*, 2017). Thermo profile is standardized for ISSR technique. The PCR thermal cycling was performed at different annealing temperatures ranging from 40.0 to 54.8°C which is different for each primer and it produced clear amplification and was used for further study.

5.5 Marker analysis

Molecular analysis can be employed for individual identification of cultivars or rootstock for determination of genetic relatedness. Unique fingerprints of particular cultivar and marker specific alleles that may serve as indicators of a particular region of the genome specific to a particular trait of horticultural importance. Thus, PCR based multiple loci marker techniques which include Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR) or microsatellites are playing important role in crop improvement (Manimekalai *et al.*, 2004). Among the different molecular markers, the ISSRs are powerful, rapid, simple, reproducible and inexpensive way to assess genetic diversity or to identify closely related cultivars in many species, including Alphonso. The potential of ISSR markers in fingerprinting is well established in mango. ISSR marker technique (Pandit *et al.*, 2007; Samant *et al.*, 2010; Archak *et al.*, 2014) have been used for analysis of mango germplasm in different regions.

The present study thus provides evidence that the ISSRs appear to be effective to explore the molecular polymorphism and to assess the genetic relationships in the Alphonso from

different locations. Using ISSR primers, it was demonstrated that all the Alphonso samples from selected 10 locations could be easily distinguished. Over all these data extends the knowledge of ISSR application as a molecular tool in mango as reported by several workers (Samant *et al.*, 2010; Gajera *et al.*, 2011; Rocha *et al.*, 2012; Archak *et al.,* 2014; Mansour *et al.*, 2014; Tomar *et al.*, 2016), who had used ISSR and other markers for molecular characterization of mango.

Marker analysis helps to understand the genetic variation present among the different 10 selected locations and within five samples of each location of Alphonso variety. The present study employed ISSR marker technique to assess genetic variations.

5.5.1 Polymorphic Information Content (PIC):

Markers that have the ability to detect high number of discernable alleles are the suitable marker for molecular characterization and genetic diversity analysis. The polymorphism information content (PIC) value of each marker, which can be evaluated on the basis of its alleles, varied greatly for all tested ISSR loci. The PIC expresses the discriminating power of the locus taking into account not only the number of alleles that are expressed, but also their relative frequencies and frequency of alleles per locus (Samant *et al.*, 2010). The PIC values calculated for the 16 ISSR primers. In this present study the highest PIC value produced by the primer UBC-876 (0.884) while the lowest PIC value given by the primer UBC-891 (0.00). Average polymorphic information content was 0.733 among the all 50 samples of Alphonso. These results are conformation with Gajera *et al.*, (2011) that is the PIC value was ranged from 0.138 to 0.398 across 20 mango genotypes. The higher PIC value indicated the informativeness of the primer. Hence the primer

UBC-876, UBC-886, UBC-818 and UBC-881 can use in future studies in the field of taxonomical and genetic resource management.

5.5.2 ISSR analysis in between ten selected locations

Sample number C1, A1, G1, J1, L1, K1, M1, D1, P1 and V1 was taken individually to characterize and to assess the genetic variation present in between Alphonso samples from selected 10 locations by using 16 ISSR primers. A total of 746 scorable DNA fragments were produced and among them 326 DNA fragments were found to be polymorphic in between these ten samples of Alphonso. Pandit *et al.*, (2007) reported the genetic diversity between 71 mango genotypes with 33 ISSR primers yielded total 420 scorable bands on amplification. The average per cent polymorphism across the 16 primers in between ten selected locations found to be 46.62 per cent which is higher than those obtained in between population studies of mango (42.86 % by Kheshin *et al.*, 2016). The product size ranged from 320 bp to 2891 bp is somewhat similar with the result of Kheshin *et al.*, 2016 (326 bp to 3125 bp). The overall range of the similarity in between ten selected locations of Alphonso was 0.559-0.733 is less than result of Kheshin *et al.*, 2016 (0.83-0.95). In the present study some fragments were uniquely identified or absent in some of the locations. These fragments are of great interest in identification of Alphonso of particular location.

5.5.2.1 Clustering analysis based on SSR markers between selected 10 locations

On the basis of analysis of ISSR scoring, the alleles were converted to binary score based on their presence (1) or absence (0). This data was used for similarity based analysis using the program Multi Variate Statistical Package (MVSP) to determine

the Jaccard's coefficient matrices i.e. estimate of similarity among the selected 10 locations.

The dendrogram based on Jaccard's similarity coefficients was constructed using UPGMA after analysis of banding patterns generated by all the samples with 16 ISSR primers. The dendrogram separated the selected locations into 2 main clusters, *viz*; I and II. Cluster I is the major cluster showing the most distant location i.e. Pawas. This location is situated at the middle of South Konkan. This region has the latitude 16°50' to 16°55' N and longitude 73°19' E. The cluster II has 2 major clusters, *viz*; IIA and IIB. Cluster IIA with Alphonso samples from 2 locations i.e. Ambivali-Kelshi and Murud from north Ratnagiri being closely related. These two locations comes under same geographical zone i.e. Tahsil- Dapoli and the genetic distance was 0.685. Cluster IIB is further divided into 2 minor clusters IIBa and IIBb. Alphonso sample from Padel and Rameshwar-Girye from same Tahsil-Deogad having the genetic distance 0.723 were included in cluster IIBa. These two locations has the latitude 16°28' N to 16°31' N and longitude 73°20' E. Remaining all locations *viz*; Adivare from Tahsil-Rajapur, Nadan-Jamsande from Tehsil-Deogad, Achara and Katta from Tahsil-Malvan, Vengurle from Tehsil-Vengurle showing close relatedness which are falling under another cluster IIBb. Vasugi *et al.,* (2012) grouped 43 mango genotypess into two major clusters, 11 varieties in cluster I whereas; 32 varieties were grouped in cluster II. The mango samples does not show a very much marked differences among themselves as the mango samples analyzed belonged to the single region i.e. South Konkan. The vicinity to sea side had a great effect on the mango samples which brought about very minor differences in genetic character

among the samples. The similar results were also obtained by Karihaloo *et al.,* (2003) who showed that a high diversity within the regions of India and confirmed that this is not surprising given that the mango is a cross-pollinated plant and selecting superior strains according to the taste among naturally produced seedlings has given birth to the commercial cultivars and the observed appreciable range of variation.

5.5.3 ISSR analysis within each location

Sixteen ISSR primers were used to characterize 50 samples of Alphonso variety. On an average maximum polymorphism percentage was found in Pawas (47.05 %) and minimum polymorphism percentage was observed in Padel (32.18 %). The overall an average maximum similarity found in between five samples of Vengurle (0.648-0.855), whereas minimum in Adivare (0.588-0.730). Bally *et al.*, (1996) was observed a genetic dissimilarity of 0.05% among 27 accessions of Kensington Pride using RAPD marker in previous studies. Addition, Singh *et al.*, 2009 detected an intracultivar variability in Banganapalli, Dashehri, and Langra cultivars of mango by using ISSRs. The dendrogram of each location separated the 5 Alphonso samples into two main clusters, *viz*: I and II. Clustering of cultivar belonging to different geographic region suggests that they might have evolved from the existing mango gene pool from which they were selected by local people to domesticate them in different areas for cultivation (Vasugi *et al.*, 2012).

For every location these Alphonso samples are separated because when cultivars from one region are grown at other region, they vary in bearing characteristics, fruit quality and time of flowering. Superior chance seedlings selected as cultivars led to fixation of very high degree of heterozygosity, leading to

high within region variation (Archak *et al.*, 2014). As with other vegetative propagated clonal crops, the differences among mango cultivars can result from epigenic modifications in response to the environment. Somatic and bud mutations also play a minor role in clonal polymorphism in woody plants. The significant variation among the trees of some clones in mango was observed with respect to fruit characteristics and tree performance (Kumar *et al.,* 2013).

The variation observed in each location due to mango may be monoembryonic and polyembryonic, based on their ability to reproduce from seeds. Most of the monoembryonic cultivars are propagated vegetatively by grafting or budding onto monoembryonic or polyembryonic seedling rootstocks. There appears to be a considerable variability reported within certain cultivars grafted onto different rootstocks, which attributed either to somatic mutations or to the influence of non-uniform monoembryonic rootstocks (Venkateswarlu, 2013).

Mango, being highly heterozygous, has a large diversity that has resulted from propagation by its seeds. The selected trees having the age more than 20 years. Since the crop is vegetative propagated, the trees of Alphonso cultivar were subjected to sub-cloning repeatedly over a period of time, across several geographical locations. This would have resulted in the production of bud sprouts or somatic mutants. Intracultivar study of genome from different locations can confirm whether there are any genetic differences among the location specific clones or not (Dinesh *et al.*, 2015). Souza *et al.,* (2011) evaluated genetic variability of mango (*Mangifera indica* L.) accessions, of which 35 originated from Brazil, six from USA, and one from India. These accessions were found to have considerable genetic

variability, demonstrating the importance of analyzing each genotype in a collection, to efficiently maintain a germplasm collection.

In Konkan region of Maharashtra where Alphonso is largely cultivated, half-sib stones will be invariably used for raising the rootstocks for grafting Alphonso variety. The rootstock effect on scion appeared to be the same, which was evident in the absence of clonal variations in scion material as clones of the Konkan region of Maharashtra (Manchekar, 2008). Due to such problems Alphonso mango has variation within location also.

This study revealed that ISSR markers are useful not only for location wise genetic relatedness identification, but also in future mango breeding programs to mango crop improvement.

CHAPTER VI

SUMMARY AND CONCLUSION

Traditionally, genetic characterization in mango has been carried out by means of morphological characters (IPGRI, 2006; PPVFRA, 2008). However, influence of environmental and developmental factors, existence of limited variation and subjective nature of evaluation of these traits have often posed problems (Adato *et al.*, 1995). Since 1990's various types of DNA markers have been employed to complement the characterization procedure (Schnell *et al.*, 2006). Mango is a cross-pollinated crop in nature. Thus, mango exhibits large genetic diversity within and among the populations. Study of genetic variation provides sufficient scientific data for germplasm management. Diversity analysis in Alphonso has been done by morphological traits, biochemical and molecular markers. Molecular markers are detectable at all stages of development and can cover the entire genome. They detect variation at DNA level, overcome most limitations of morphological and biochemical markers.

For this analysis, mango (Alphonso) samples were selected from different location of South Konkan. DNA was extracted following the protocol of Doyle and Doyle (1990). Purified DNA was used for amplification with ISSR primers. Amplification products were then separated by agarose gel electrophoresis. The gel was photographed under Gel documentation system (UV transilluminator) and were scored for the presence (1) and absence of a band (0). This binary matrix of (1) & (0) was fed to Multi Variate Statistical Package (MVSP) software to generate similarity matrix. Further, cluster analysis was performed using UPGMA.

- Standardization of buffer constituents for DNA isolation which is the prerequisite for ISSR-PCR reactions showing optimum results at 0.900 g glucose, 0.100 g PVP, 0.040 g sodium bisulphite, 0.050 g Sodium lauryl sulphate, 500 µl sarcosyl.
- Modifications in extraction procedure like decrease in sample size, twice the volume of isopropanol alcohol and washing with 70% ethanol yielded good quality DNA.
- Dilutions of crude extract of DNA of all samples gave amplifiable quantity of DNA.
- Modification in PCR parameters like PCR master mixture and thermo profile showed clear and specific banding pattern.
- Sixteen ISSR primers were used for the study.
- The polymorphic information content (PIC) values ranged from 0.00 to 0.884 with an average PIC value of 0.733 per primer. The ISSR primer, UBC-876 revealed highest (0.884) PIC value; whereas the primer UBC-891 revealed the lowest (0.00) PIC value.
- Maximum 7 alleles were produced by the primer UBC-876, UBC-881 and UBC- 886 whereas the primers UBC-884, UBC-857, UBC-853 and UBC-817 produced minimum 2 alleles.
- ISSR primers showed 46.62 per cent average polymorphism in between ten selected locations. On an average maximum polymorphism percentage was found in Pawas (47.05 %) and minimum polymorphism percentage was observed in Padel (32.18 %). The overall range of similarity between selected 10 locations was ranged from

0.559 to 0.733. The overall an average maximum similarity found in between five samples of Vengurle (0.648-0.855), whereas minimum in Adivare (0.588-0.730).

- The dendrogram separated 10 locations and five samples of each location of Alphonso into two main clusters.

Conclusion

This study indicated that ISSR Markers are suitable for the molecular analysis of Alphonso from different locations of south Konkan. In this present study molecular signature of Alphonso from different locations was established. The ISSR analysis revealed the moderate polymorphism in Alphonso. The results of present study indicated the efficiency of ISSR markers in investigating the genetic variation at molecular level. Such study is important for detecting the distinctness of the same variety from different geographical locations and also for identification of desirable samples and it's utilization for further breeding program. But this variation indicates that there is need to maintain original genotype of Alphonso because genetic variation obtained within different plant samples of same location is not expected.

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Molecular analysis of Mango (*Mangifera indica* L.) Cv. Alphonso from different locations of South Konkan

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Student's Name: Patil Vinayak Babanrao Research Guide**:** Dr. S. V. Sawardekar

ABSTRACT

The present study was carried out with an objectives to establish the molecular profile of Alphonso from different locations of south konkan and to analyze the variation between them.

The DNA was extracted from tender leaf samples collected from ten different selected locations (5 samples from each location). The standardization of buffer constituents for DNA isolation was carried out. Five components of the extraction buffer were standardized by using rapid method. The results obtained using 0.900 g glucose, 0.100 g PVP, 0.040 g sodium bisulphite, 0.050 g sodium lauryl sulphate, 500 µl sarcosyl were most suitable. Modifications in extraction procedure resulted into better and clear banding pattern when subjected to PCR analysis. Modification in PCR parameters like PCR master mixture and thermo profile showed clear and specific banding pattern.

The average per cent polymorphism showed by 16 ISSR primers in between ten locations were 46.62 per cent. In ISSR marker, the overall range of similarity ranged from 0.559 to 0.733. The polymorphic information content (PIC) values ranged from 0.00 to 0.884 with an average PIC value of 0.733 per primer. UPGMA grouped 5 alphonso samples of each location into two main clusters.

Substantial average polymorphism was detected by ISSR primers among five samples from Achara (42.83 %), Adivare

(42.39 %), Rameshwar-Girye (42.73 %), Nadan-Jamsande (46.77 %), Katta (44.22%), Ambivali-Kelshi (44.68 %), Murud (46.29 %), Padel (30.45 %), Pawas (47.05 %) and Vengurle (42.67 %).

The ISSR analysis revealed the moderate polymorphism in Alphonso. Study indicates that genetic variation was found between selected ten locations and also within five samples of Alphonso of each location. Such study is important for detecting the distinctness of the same variety from different geographical locations and also for identification of desirable samples and it's utilization for further breeding program.

Keywords: Mango, Polymorphism, ISSR, Marker.

INTRODUCTION

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MATERIAL AND METHODOLOGY

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