

**“MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR  
CHARACTERIZATION OF DIFFERENT GENOTYPES  
OF *ANNONA* SPECIES”**

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2015**

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**“MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR  
CHARACTERIZATION OF DIFFERENT GENOTYPES  
OF ANNONA SPECIES”**

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**OF**

**Master of Science**

**(Agriculture)**

**IN**

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# **Morphological, Biochemical and Molecular Characterization of Different Genotypes of *Annona* species”**

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## **ABSTRACT**

*Annona* belongs to the family *Annonaceae*, which is mostly known for its edible fruits. *Annona* fruits are of the world's best tasting fruits due to their sweet, delicious, creamy flesh and fragrant flavor when fully ripe. Fruits contain proteins, fatty acids, fibres, carbohydrates, vitamin C, antioxidants and minerals such as calcium, phosphorous and potassium. Leaves and seeds are also potential source of secondary metabolites and antioxidants. A good amount of oil is present in seeds which can be useful for industrial purpose.

Looking at the importance of *Annona* species, the present study was conducted with the aim of assessment of genetic variability in different genotypes of *Annona* species by morphological parameters, estimation of various biochemical parameters from fruits and seeds of *Annona* species and to study the molecular

polymorphism in different genotypes of *Annona* species through RAPD and SSR markers.

The study included twenty genotypes of *Annona* (five species, twelve cultivars and four hybrids), of which nine matured and fruiting genotypes were evaluated for morphological and biochemical characterization using CRBD design with three replications, whereas all twenty genotypes were used in molecular study for genetic diversity analysis.

Result indicated the superiority of *A. atemoya* (Interspecific hybrid) due to high mean value of its leaf length, leaf width, petiole length, petal length, petal width, fruit width, fruit weight and fruit volume, whereas *A. cherimola* was superior for higher number of fruits per plant. These two genotypes can be exploited in the improvement of *Annona* species for higher yield. Variability component study revealed that leaf length, leaf width, petal length, petal width, fruit length, fruit width, fruit weight, fruit volume, number of fruits per plant, seed length, seed width, number of seeds per fruit and hundred seed weight possessed high heritability coupled with high genetic advance, which indicated that these characters are mainly governed by additive gene action, hence simple selection will be very effective for crop improvement. Correlation study between different morphological parameters suggested the strong genotypic and phenotypic association of fruit yield per plant with leaf length, leaf width, fruit length, fruit width, fruit weight, fruit volume and

number of fruits per plant so these traits would be given due weightage for direct improvement of fruit yield in *Annona* species.

*A. Squamosa* var. GJCA-1, Balanagar and Sindhan possessed significantly higher amount of carbohydrates, Total soluble sugars, proteins, edible fibres and ascorbic acid as compared to other genotypes, suggesting a good choice for fruit breeders and consumers. *A. muricata* contained significantly higher amount of seed oil, antioxidant activity (seeds and pulp) and various phytochemicals, indicating a potential scope for fuel industries and pharmaceutical industries, respectively. Correlation study between different biochemical parameters suggested the strong genotypic and phenotypic association of carbohydrates with TSS, reducing sugars and fibre content and ascorbic acid with antioxidant activity, which means quality improvement in *Annona* species for simultaneous two or more parameters can be possible.

Molecular study revealed the existence of considerable genetic diversity amongst different genotypes of *Annona* species. RAPD markers were found more informative as compared to SSR for discriminating different *Annona* genotypes; therefore, RAPD can be better exploited for identification of different genotypes of *Annona* species, which will be ultimately useful in the improvement of the crop.

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## **CERTIFICATE**

This is to certify that the thesis entitled “**Morphological, Biochemical and Molecular Characterization of Different Genotypes of *Annona* species**” submitted by **Mr. Hirdayesh Anuragi (Reg. No.: 04-2124-2013)** in partial fulfillment of the requirement for the award of the degree of **Master of Science (Agriculture)** in the subject of **Genetics and Plant Breeding** of Anand Agricultural University, is a record of bonafied research carried out by him under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

**(H. L. Dhaduk)**

Major Advisor

**Place:** Anand

**Date:** 15/07/2015

# DECLARATION

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This is to declare that the whole of the research work reported here in for the partial fulfillment of the requirement for the degree of **Master of Science in Genetics and Plant Breeding** by me under the guidance and supervision of **Dr. H. L. Dhakuk**, Associate Professor, Department of Genetics and Plant Breeding, BACA, AAU, Anand. The thesis has not previously formed the basis for the award of any degree, diploma or other similar titles.

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*“Gratitude is the most exquisite form of money”*

*Acknowledgement is written at last, placed at first and read at least, but still it is the golden opportunity to thank one and all who are responsible in completion of this mite of mine to science.*

*My post graduate journey is now almost near to an end. It has been a very good experience for me and I really learnt so many things out of it. As no scientific endeavor is a result of an individual's efforts so it is the time to look back on the path travelled during this endeavor and to remember the faces and spirits behind the action with a sense of gratitude.*

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Place: Anand.

Date:

(Hirdayesh Anuragi)

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## LIST OF ABBREVIATIONS

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|                       |                                    |
|-----------------------|------------------------------------|
| 1. %                  | Percentage                         |
| 2. °C                 | Degree Celsius                     |
| 3. µg                 | Microgram                          |
| 4. µl                 | Micro liter                        |
| 5. bp                 | Base pair                          |
| 6. CD                 | Critical difference                |
| 7. CTAB               | Cetyl Trimethyl Ammonium Bromide   |
| 8. CV                 | Coefficient of variaton            |
| 9. DNA                | Deoxyribonucleic acid              |
| 10. <i>e.g.</i>       | Example                            |
| 11. EDTA              | Ethylene Diamine tetra Acetic Acid |
| 12. <i>et al.</i>     | Co workers                         |
| 13. <i>etc.</i>       | Etcetera                           |
| 14. gm                | Gram                               |
| 15. HCl               | Hydrochloric acid                  |
| 16. kb                | Kilo base                          |
| 17. M                 | Molar                              |
| 18. mg                | Milligram                          |
| 19. MgCl <sub>2</sub> | Magnesium Chloride                 |
| 20. Min               | Minutes                            |
| 21. ml                | Millilitre                         |
| 22. mM                | Millimolar                         |

|             |                                                  |
|-------------|--------------------------------------------------|
| 23. Mol wt. | Molecular weight                                 |
| 24. N       | Normality                                        |
| 25. NaCl    | Sodium chloride                                  |
| 26. ng      | Nano gram                                        |
| 27. ng/L    | Nanogram per litre                               |
| 28. nm      | Nanometer                                        |
| 29. No.     | Number                                           |
| 30. OD      | Optical density                                  |
| 31. PCR     | Polymerase Chain Reaction                        |
| 32. pH      | Negative logarithm of hydrogen ion concentration |
| 33. PIC     | Polymorphism Information Content                 |
| 34. pmole   | Pico mole                                        |
| 35. PVP     | Polyvinyl pyrrolidone                            |
| 36. RAPD    | Randomly amplified polymorphic DNA               |
| 37. rpm     | Revolution per minute                            |
| 38. SAHN    | Sequential Agglomerative Nested Cluster          |
| 39. SEm     | Standard error of mean                           |
| 40. SSR     | Simple sequence repeats                          |
| 41. TBE     | Tris-Borate EDTA                                 |
| 42. TE      | Tris EDTA                                        |
| 43. TSS     | Total soluble sugars                             |
| 44. UPGMA   | Unweighted Paired Group Mathematical             |
| 45. viz.    | Namely                                           |

## I. INTRODUCTION

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India is endowed with a diverse agro-climatic condition permitting the cultivation of large number of tropical, subtropical and temperate fruit crops. Fruit cultivation in India is a source of high economic returns to the farmers, prominent business sector for exporting merchandise and earning a very good amount of international revenue for the country. Research efforts and concern for improving the productivity has resulted in many fold increase in area and production. In late fifties, estimated fruit production was only 7.4 million tonnes from 1.22 million hectares with the productivity of 6.06 million tonnes per hectare and in contrast, during 2012-13 a total of 81.28 million tonnes fruits were produced from 6.98 million hectares with the productivity of 11.64 million tonnes per hectare. Currently, India is the second largest producer of fruit crops after China and around 10 percent of the world fruit production is accounted by India.

*Annona* belongs to the family *Annonaceae*. It is a multipurpose tree and fruit is the edible and economic part. It is considered as one of the most delicious and nutritionally valuable fruit for table purpose. *Annona* fruits have edible, soft, juicy sugary pulp with mild flavor with slight acidity and are used in ice cream, confectionery and certain milk products. Other than fruits, tree is also a source of various other medicinal and industrial products.

*Annona* fruit is a useful food because it contains proteins, fatty acids, fibres and is also an excellent source of energy as being high in carbohydrate content

(Bueso, 1980; Leal, 1990; Lizana and Reginato, 1990). In addition, fruit also contains vitamin C and minerals such as calcium, phosphorous and potassium. Leaves and seeds of *Annona* are good source of secondary metabolites and antioxidants. Seed contains good amount of oil which can be used for industrial purpose.

The genus name *Annona* derives from the Latin word “anon” meaning annual harvest (Lizana and Reginato, 1990). *Annonaceae*, which is included within the order Magnoliales, is one of the largest tropical and subtropical families of trees, shrubs, and lianas. It has about 135 genera and 2500 species distributed worldwide.

The origin of most of *Annona* species is South America and the Antilles, however wild soursop (*A. muricata*) is thought to have originated in Africa. The current distribution of important species covers almost all continents, with soursop and sugar apple showing the widest distribution, mainly in tropical regions. In India, there is a very large, diverse population of sugar apple, and its commercial importance is so great that some botanists have considered it to be a native fruit of that country. However, this is a secondary centre of diversity, created during the last 500 years (Pinto *et al.*, 2005).

Chromosome numbers among *Annona* species do not vary significantly and most of the *Annona* species possess a chromosome number of  $2n = 2x = 14$  and  $16$ , except for *A. glabra* which is a tetraploid species ( $2n=4x=28$ ). The diploid

chromosome number for *A. squamosa* and *A. cherimola* is 14, although  $2n = 16$  has also been reported (Darlington and Wylie, 1956).

Three important generas in the *Annonaceae* (*Annona* L., *Rollinia* St.-Hil., and *Asimina* Adans.) contain species with edible fruit, such as cherimoya (*A. cherimola*), sugar apple (*A. squamosa* L.), atemoya (a hybrid between *A. cherimola* and *A. squamosa*), soursop (*A. muricata* L.), custard apple (*A. reticulata* L.). All other species of *Annonaceae* were in an incipient stage of domestication, however recently some species of genus *Annona* achieved great importance in the orchards.

**Table 1.1 Botanical names and synonyms of different *Annona* species**

| S.No | Botanical name                           | Synonyms                                |
|------|------------------------------------------|-----------------------------------------|
| 1.   | <i>A. cherimoya</i> Mill. ( $2n=2x=14$ ) | Cherimoya Cherimola                     |
| 2.   | <i>A. reticulata</i> L. ( $2n=2x=14$ )   | Ramphal, Custard apple, Bullock's heart |
| 3.   | <i>A. muricata</i> L. ( $2n=2x=14$ )     | Laxmanphal, Soursop                     |
| 4.   | <i>A. atemoya</i> Mabb. ( $2n=2x=14$ )   | Hanumanphal, Pineapple sugarapple       |
| 5.   | <i>A. squamosa</i> L. ( $2n=2x=14$ )     | Sitaphal, Sugar apple, Sweetsop         |

Pinto *et al.* (2005)

In general, the *Annona* are shrubs or small trees, whose heights vary from 5 to 12 meters depending on several factors such as type of species, climate, soil and crop management. They are erect or somewhat spreading in habit, with grey brown bark, often rough and corrugated. Stems are ferruginous to grayish and tomentose when young but later become glabrous.

In *Annona* species, flowering starts when the plant is three to four years old, although it may occasionally occur earlier depending upon environmental condition. Anthesis (flower opening) starts slowly with the separation of the apex of the external petals and takes around 6 to 8 hours to complete.

Flowers are hermaphrodite, usually somewhat fragrant, solitary or fascicle with 2 to 4 flowers with three green sepals and 6 petals arranged into two verticils. Flowers have several conglomerated spirally arranged stamens below and around an upper globose (conical) shaped dome of numerous united carpels which have one ovule each. This makes flower protogynous, favouring the formation of hybrids over self-pollination (Gazit and Eisenstein, 1985). Cross pollination is mainly carried out by insects like nitidulid fruit beetles (Gazit *et al.*, 1982) or sometimes by wind.

*A. squamosa*, locally known as ‘Sitaphal’ is the most popular *Annona* species in India grown in about 30,000 hectares mainly in the states of Andhra Pradesh, Gujarat, Assam, Tamil Nadu and some parts of central India, with an approximate annual production of 136,000 tonnes.

*Annona* trees can give an average fruit yield of 50-100 kg/tree and the commercial life of a tree is about 15 years. The trees are very easy to cultivate, require comparatively little care and do not suffer from serious pests and diseases infestations.

In Indian agriculture, drought is the single most important factor which limits the yield and quality of traditional field crops. Thus, shift from rain fed field crops to growing of fruit orchards in the dry lands will have beneficial consequences both economically and environmentally. In order to achieve increased production in dry and marginal lands, *Annona* species which are hardy and deciduous in nature, seems to be very suitable with minimum inputs.

Genetic variation is essential for long term survival of species and it is a critical feature in conservation. To expedite the crop improvement programme, it is necessary to identify and trap the natural variability through surveys and the variability should be conserved *ex situ* and *in situ* to utilize in the further hybridization programmes. Proper study is necessary to build up the understanding of this crop so that sustainable efforts can be put forward to enhance production, accelerate research and develop capabilities to make *Annona* as a profitable enterprise amongst fruit growers of the in India.

Study at morphological, biochemical and molecular level can provide us the basis for identification and differentiation of various species and their cultivars which can be further used in planning for the improvement of the crop.

### **Morphological studies**

In the past few years, morphological traits have been used as primary tools to identify and characterize germplasm resources. Unfortunately, morphological characters are highly influenced by environmental factors and the problems with

ambiguity are frequent. In contrast to morphological markers, biochemical and molecular markers are emerged as powerful tools for proper characterization of germplasm diversity. In case of *Annona* species few studies based on morphological characters have been done in recent past (Pinto *et al.*, 2005; Folorunso *et al.*, 2006 and Folorunso *et al.*, 2007).

### **Biochemical studies**

Information on the nutrient composition of locally available foods especially fruits is of immense importance because of the need to supply foods according to their nutritive values to the people. Dietary supplements are now made using different fruits, vegetables and also herbs in order to provide natural curative as well as preventive methods for combating diseases and poor health. Because of the importance of *Annona* fruits as valuable food and medicinal resource, many studies have been undertaken to identify, characterize and differentiate various locally grown cultivars of *Annona* in terms of their proximate fruit composition, antioxidant activity and phytochemical properties of the fruits.

Studies on the proximate fruit composition of various genotypes of *Annonaceae* have been previously reported by Onimawo, 2002; Amoo *et al.*, 2008; Kimbonguila *et al.*, 2010; Pareek *et al.*, 2011; Kulkarni *et al.*, 2013 and Boake *et al.*, 2014; screening of antioxidant activity by Mariod *et al.*, 2012 and phytochemical activity by Gowdhami *et al.*, 2014.

**Molecular markers**

In addition to morphological and biochemical markers, genetic markers can also be used for differentiating various genotypes. They provide genetic basis of diversity, which is more accurate and reliable as compared to morphological and biochemical markers.

In case of *Annona*, series of markers such as Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSRs) or microsatellites and Amplified Fragment Length Polymorphism (AFLP) have been developed to study the genetic diversity. Few attempts related to RAPD by Ronning *et al.*, 1995; Bharad *et al.*, 2009; SSR by Escibano *et al.*, 2004; Kwapata, 2007 and AFLP Rahman *et al.*, 1998; Zhichang *et al.*, 2011, markers have been made to study the genetic diversity of *Annona* species. However, very scanty information is available on use of both biochemical and molecular markers together for diversity analysis in *Annona* species. So, there is a strong need to make attempts for generating information on different aspects of various *Annona* species and their utilization in crop improvement.

Therefore, keeping in view all above aspects, the present study on **“Morphological, biochemical and molecular characterization of different genotypes of *Annona* species”** was undertaken with the following objectives;

**Objectives:**

- ❖ Assessment of genetic variability in different genotypes of *Annona* species by morphological parameters.
- ❖ Estimation of various biochemical parameters from fruits and seeds of different genotypes of *Annona* species.
- ❖ Study the molecular polymorphism in different genotypes of *Annona* species through RAPD and SSR markers.

## II. REVIEW OF LITERATURE

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*Annona* species are not much exploited in terms of modern research; however few attempts have been made to study the importance of *Annona* species. Study of various morphological parameters related to leaf, flower, fruit and seeds can be useful for identification and differentiating of different species and varieties of *Annona*. Knowledge about various quality parameters such as moisture content, total carbohydrates, total soluble sugars, protein content, fibres and ascorbic content *etc.* can help in improving the quality of the crop. Study of molecular basis can facilitate in understanding genetic relationship among the genotypes.

The present review is an attempt to bring together some of the findings on morphological, biochemical and molecular aspects of *Annona* species and to develop a common understanding for utilizing the ample scope in this field for various uses.

### 2.1. Morphological characterization

Morphological study can be useful in taxonomy for identification and classification of plants at intra-specific and interspecific level. In case of *Annona*, knowledge of characters related to leaf, flower, fruit and seed morphology is very important for identification and differentiating local cultivars. This can help in the improvement of the crop. Few attempts have been made in search of these qualitative and quantitative characters which can be of taxonomic importance. Some of them are as follows;

Pinto *et al.* (2005) in UK published a monograph having detailed information on five important *Annona* species that is *A. squamosa*, *A. cherimola*, *A. reticulata*, *A. muricata* and a wild species *A. diversifolia*. They studied tree, branches, leaves, flowers, fruits and seed characteristics for all above mentioned species. All other aspects were also explained in order to provide opportunity for farmers, researchers to exploit the *Annona* species.

Folorunso *et al.* (2006) studied four species of *Annona* found in Nigeria in search of intra-generic characters which may be of taxonomic value in the identification and classification of the genus. Plant parts like leaves, fruits, flowers, seeds were studied for their qualitative and quantitative characters.

Folorunso *et al.* (2007) studied the leaf architecture in four species of *Annona* characterized as important under-utilized species. Leaf lamina balance, leaf form, leaf apex, leaf base, leaf margin, leaf texture were studied as the architectural features. More intra-generic relationships among the *Annona* have been reported along with characters of taxonomic value in the identification and classification of the genus. Biochemical basis was also utilized to differentiate the species. *A. reticulata* and *A. squamosa* were found nutritionally more valuable with more shelf life.

## **2.2. Biochemical characterization**

Biochemical study included proximate analysis, antioxidant activity and phytochemical screening for secondary metabolites. The research works done so far, is summarized as follows;

### 2.2.1 Proximate analysis

Proximate composition included the analysis of biochemical quality parameters of fruits. Keeping quality is the major issue in *Annona* fruits and moisture content is one of the most important factors which actually decides the quality and storability of the fruit.

Moisture content is inversely related to dry matter and has more effect on the keeping quality of the fruit. The fruit with high moisture content may deteriorate early; therefore it is very important to know the moisture content in order to make a reasonable and accurate prediction of the possible storage life the fruits. And ash content decides the total amount of mineral elements present in the fruit. Carbohydrates perform numerous roles in the living organisms. Polysaccharides serve for the storage of energy and as structural components. In addition to this, they are also important for immune system of the organisms.

Proteins are essential component of our diet because they are the structural components of body tissues and used in many biological processes (enzymes, antibodies and DNA). Fibres or roughage are indigestible portion of food which is derived from the plants. High Fibre content increases the digestibility so such fruits with more fibre content are always preferred. Phenolic compounds occur in all fruits as secondary metabolites, hence they are the components of human diet. Phenolic compounds are important in oxidation processes.

Titrateable acidity is the measure of amount of acid present in the solution eg. tartaric, malic and citric acid. Such acids play significant role in taste, colour and microbial stability of the juice. In many fruits acidity is an important factor in determining maturity. Vitamin C is a water soluble vitamin, means our body does not store it. It is needed for growth and repair of tissues in all parts of body and also for healing wounds. It is also an antioxidant and block damages caused by free radicals. Some important reviews of fruit proximate composition are briefly summarized as follows;

**Table 2.1 Reviews related to proximate composition in *Annona* species**

| S.No. | <i>Annona</i> species                                                                               | Parameters<br>(per 100 gm pulp and seed)                                                                                                                                                                                                                                                                                                                                                                                           | References                      |
|-------|-----------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|
| 1.    | <i>A. muricata</i>                                                                                  | <b>Seed:-</b> 8.5% Moisture, 13.6% ash, 20.5% fat, 47% carbohydrates, 2.4% crude protein, 0.79% titrateable acidity.<br><b>Pulp:-</b> 81% moisture, 8% Crude fibre, 1.23% protein, 3.43% titrateable acidity.                                                                                                                                                                                                                      | Onimawo <i>et al.</i><br>(2002) |
| 2.    | <i>A. cherimola</i><br><br><i>A. reticulata</i><br><br><i>A. muricata</i><br><br><i>A. squamosa</i> | <b>Pulp:-</b> 77.3g moisture, 18.42g carbohydrates, 0.7g ash, 1.6g protein, 1.64g fibre. 11.5mg ascorbic acid.<br><b>Pulp:-</b> 75.8g moisture, 18.7g carbohydrates, 0.95g ash, 1.85g protein, 2.55g fibre, 30mg ascorbic acid.<br><b>Pulp:-</b> 81.25g moisture, 17.25g carbohydrates, 0.61g ash, 1.0g protein, 0.86g fibre, 29.4mg ascorbic acid.<br><b>Pulp:-</b> 72.6g moisture, 19.6g carbohydrates, 0.7g ash, 1.68g protein, | Pareek <i>et al.</i><br>(2011)  |

|     |                      |                                                                                                                                                           |                                     |
|-----|----------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------|
|     |                      | 1.4g fibre, 37.38mg ascorbic acid.                                                                                                                        |                                     |
| 3.  | <i>A. cherimola</i>  | <b>Seed:-</b> 17.36% protein, 29.39% fat.<br><b>Pulp:-</b> 4.48% protein, 1.56% fat, 43.38mg ascorbic acid, 0.023% acidity, 27.25% TSS.                   | Amoo <i>et al.</i><br>(2008)        |
| 4.  | <i>A. muricata</i>   | <b>Seed:-</b> 7.7% moisture, 8.5% protein, 40% fat, 34.1% carbohydrates.                                                                                  | Kimbonguila <i>et al.</i><br>(2010) |
| 5.  | <i>A. squamosa</i>   | <b>Peel:-</b> 3.73% moisture by Karl Fisher method and 3.96% moisture by loss on dry method.                                                              | Kulkarni <i>et al.</i><br>(2013)    |
| 6.  | <i>A. muricata</i>   | <b>Pulp:-</b> 2.44 to 23.24% carbohydrates, 2.63 to 6.71% proteins, 11.50gm fibre content and 20.33 to 63.67mg ascorbic acid.                             | Boake <i>et al.</i><br>(2014)       |
| 7.  | <i>A. muricata</i>   | <b>Pulp:-</b> 73.1 to 82.1% moisture content, 0.87mg ash, 3.11 to 6.09mg crude fibre, 0.10 to 1.25% ca titratable acidity and 19.7 to 34mg ascorbic acid. | Othman <i>et al.</i><br>(2014)      |
| 8.  | <i>A. reticulata</i> | <b>Pulp:-</b> 19.92 to 21.26% total sugars, 11.98 to 17.88% reducing sugars, 12.31 to 18.71mg total phenols.                                              | Benkeblia <i>et al.</i><br>(2014)   |
| 9.  | <i>A. squamosa</i>   | <b>Seed:-</b> 26.8% oil content.                                                                                                                          | Mariod <i>et al.</i><br>(2010)      |
| 10. | <i>A. squamosa</i>   | <b>Seed:-</b> 22.2% crude fatty oil.                                                                                                                      | Rana<br>(2015)                      |

### 2.2.2 Antioxidant activity

Antioxidants are the molecules that inhibit the oxidation of other molecules. Although oxidation reactions are crucial for life, they can cause damage to tissues. Presence of antioxidants is needed to prevent the activity of free radicals and so reduce the damage caused by them. In *Annona* few studies related to analysis of antioxidant activity have been done, some of them are;

Mariod *et al.* (2010) studied antioxidant activity of *Annona squamosa* (leaves, bark, roots and seed cake) and *Catunaregam nilotica* (leaves, bark and seedcake) using oxygen radical absorbance capacity, MTT assay, DPPH assays and polyphenol content. They found that *C. nilotica* and *A. squamosa* extracts displayed antioxidant activities, with IC<sub>50</sub> values ranging from 7.81 to 62.5 µg/ml and from 7.81 to 125.0 µg/ml, respectively using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The total phenolic contents were 171.5, 170.4, 169.5, and 167.9 g/kg plant extract as GAE for *A. squamosa* roots, *C. nilotica* bark, *C. nilotica* leaves, and *A. squamosa* bark, respectively using Folin-Ciocalteu method.

Vijayaraghavan *et al.* (2013) evaluated the leaf extracts of *Chromolaena odorata* and seed extract of *A. squamosa* for antioxidant activities by DPPH radical scavenging assay and found maximum antioxidant activity in ethanolic leaf extract of *C. odorata* and seed extract of *Annona squamosa* followed by others accessions with different solvents. This revealed that these plants are of therapeutic potential due to their high free radical scavenging activity.

### 2.2.3 Secondary metabolites

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of an organism but often play a significant role in plant defense against various insect pest and diseases. Some attempts for studying secondary metabolites in *Annona* include;

Savithramma *et al.* (2011) performed phytochemical screening of eighteen important medicinal plants including *A. squamosa* and *A. reticulata* and phytochemical analysis confirmed the presence of various phytochemicals like saponins, terpenoids, steroids, anthocyanins, coumarins, fatty acids, tannins, leuco-anthocyanins and emodins. The results suggested that *Annona* contains steroids, terpenoids and emodins.

Soni *et al.* (2011) carried out phytochemical screening and qualitative thin layer chromatographic separation of secondary metabolites from leaves of *A. squamosa*. They found that the most of the biologically active compounds like tannins, saponins, alkaloids and flevonoids were present in petroleum ether, chloroform, ethanol, methanol and aqueous extracts of leaves.

Florence *et al.* (2014) performed phytochemical screening of selective members of *Annonaceae* using organic solvent extract and confirmed the presence of alkaloids, carbohydrates, coumarins, flavonoids, glycosides, phenolic compounds, phytosterols, proteins, quinones, saponins, steroids and terpenoids.

Biba *et al.* (2013) studied *A. squamosa* seed extracts for their phytochemical constituents, total phenolics and flavonoid contents using petroleum ether, chloroform, ethyl acetate and methanol extracts and found moderate amounts of phytochemicals such as flavanoids, coumarines, alkaloids, terpenoids and their correlation with the possible significant medical potential of the plant.

Vijayaraghavan *et al.* (2013) investigated leaf extract of *C. odorata* and seed extract of *A. squamosa* using different solvents for phytochemical composition and revealed the presence of active ingredients such as glycosides, steroids, Saponins, Phenols, Flavonoids, Terpenoids and Tanins.

## **2.3. Molecular characterization**

### **2.3.1. RAPD markers**

Ronning *et al.* (1995) studied various cultivars of *Annona* using RAPD markers and found that *A. cherimola* ‘Campa’ and ‘Jete’, *A. squamosa* ‘Lessard’, and *A. atemoya* ‘Ubranitzki’, ‘Malali’, and ‘Kaspi’ resulted in very distinctive patterns, indicating that RAPD markers, may be an efficient method of fingerprinting genotypes within and between *Annona* species. All 15 RAPD primers generated repeatable and polymorphic patterns. An F<sub>1</sub> population of ‘Jete’ × ‘Lessard’ was analyzed to determine the inheritance of the RAPD banding patterns.

Bharad *et al.* (2009) studied 11 morphologically different genotypes of sugar apple (*A. squamosa* L.) for diversity analysis using isozyme and RAPD markers. Four RAPD primers yielded a total of 26 scorable bands, of which 19 were

polymorphic. RAPD analysis revealed that three genotypes viz., AKCa 05, AKCa 07 and AKCa 10 were divergent and the rest of eight genotypes were almost similar to each other and these 11 genotypes were grouped into three distinct groups.

Guimaraes *et al.* (2013) studied the genetic diversity in a collection of 64 sugar apples from different municipalities in northern Minas Gerais using RAPD primers. 20 RAPD primers generated 167 fragments, of which 48 were polymorphic (28.7%) producing an average of 2.4 polymorphic fragments per primer. Low percentage of polymorphism (< 29%) was observed by using the set of primers indicating low level of genetic variation among the 64 accessions evaluated. Genetic relationships were estimated using Jaccard's coefficient of similarity. The dendrogram revealed five clusters. In summary, RAPD showed a low percentage of polymorphism in the germplasm collection.

### **SSR markers**

Escribano *et al.* (2004) in Spain developed Fifteen polymorphic microsatellites using a CT/AG-enriched genomic library of *A. cherimoya* cv. Fino de Jete and screened these SSRs on 23 genotypes from different geographical areas. This was the first set of microsatellites developed in *Annonaceae*. Fourteen of the microsatellites detected a single locus and only one detected two loci. The number of alleles in the single locus microsatellites ranged from two to six and expected heterozygosity ranging from 0.17 to 0.61. Most of the simple sequence repeats were transferable to other species in the family.

Kwapata *et al.* (2007) evaluated nine populations for genetic diversity using microsatellites (SSRs) developed in *A. cherimola*. A total of 23 alleles were detected in the populations studied and genetic diversity parameters revealed high levels of heterozygosity with 4.0 to 14 alleles per locus and the populations were genetically different by 19%. It demonstrated the association between genetic and geographical distance in the species indicating that large-scale geographical and ecotypic differentiation was reflected by the SSR markers. The study has revealed evidence of application of SSR markers from *A. cherimola* towards genetic fingerprinting of *A. senegalensis*.

Escribano *et al.* (2008) have developed Fifty-two single locus polymorphic microsatellites using two genomic libraries digested with *HaeIII* and *RsaI* of *A. cherimoya* cv. Fino de Jete enriched in CT/AG repeats. A total of 222 alleles were detected with the selected SSRs. The observed and expected heterozygosities ranged from 0.08 to 0.73 and from 0.20 to 0.84, respectively. Most of the SSRs were transferable to other species in the *Annonaceae*. A set of 20 microsatellites was selected to facilitate the exchange of data among laboratories.

### III. MATERIALS AND METHODS

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The present investigation on “**Morphological, Biochemical and Molecular Characterization of Different Genotypes of *Annona* species**” was carried out during 2013-15 at different places like Morphological Characterization at Horticulture Farm, Department of Horticulture, Biochemical Characterization at Department of Biochemistry, Molecular Characterization at Department of Agricultural Biotechnology, Centre of Excellence in Biotechnology, AAU, Anand. The materials required and methodologies followed in this study are summarized and presented as follows;

#### 3.1 EXPERIMENTAL SITE

##### 3.1.1 Geographical location

Geographically, Anand is situated at the 22° 35' North latitude and 72° 56' East longitude with an altitude of 45.11 meters above the mean sea level.

##### 3.1.2 Climatic condition

Generally monsoon of middle Gujarat is warm and moderately humid. It commences by the middle of June and ends in middle September. An average rainfall of this tract is about 762 mm. Monsoon in this area is often erratic and uncertain both in respect to total rainfall and uneven distribution. Winter is fairly cool and dry while summer is quite hot and dry. The temperature rises up to 42 to 45° C during summer.



The meteorological data on maximum and minimum temperature as well as relative humidity were recorded at meteorological observatory at B. A. College of Agriculture, A. A. U., Anand during 2014 (Appendix-III).

## **3.2 EXPERIMENTAL MATERIALS**

### **3.2.1 Source of genotypes**

The experimental materials used for present study were procured from the *Annona* orchard maintained at Horticulture farm, B. A. College of Agriculture, AAU, Anand.

### **3.2.2 List of genotypes**

The experimental material was consisted of twenty different genotypes of *Annona*, out of which only nine genotypes are matured enough and producing fruits, therefore these nine genotypes were used for morphological and biochemical assessment, whereas all twenty genotypes were used for molecular diversity analysis (Table 3.1).

## **3.3 CHEMICALS, GLASS WARES AND INSTRUMENTATIONS**

### **3.3.1 Chemicals and Reagents**

All the chemicals and reagents used in the experiments were of molecular and analytical grade with higher purity purchased from commercial suppliers like Amresco Solan Industry Pkwy, Solan., Hi Media laboratories Pvt. Ltd., Mumbai., Biogene, Reagents Inc. USA, Thermo scientific, Merck (India) Ltd, Mumbai.





### **3.3.2 Glasswares and polywares**

All glasswares and poly wares were obtained from standard commercial companies like Borosil, J-SilAxygen, USA., Corning Incorporated, NY., Eppendorf AG, Germany etc. All the glass wares were cleaned by using lab wash or detergent and washed with tap water and finally rinsed with distilled water. Glass wares were dried in oven before use. All poly wares like micro tips and Eppendorf tubes were autoclaved before use.

### **3.3.3 Equipments and Instruments**

The equipments and instruments like Weighing balance, Centrifuge, Hot water bath, Microwave oven, pH meter, Refrigerator, UV spectrophotometer and Soxhlet apparatus, Gel electrophoresis unit, Gel documentation unit, Thermal cycler, NanoQuant and Hot dry bath were used for biochemical and molecular study.

## **3.4 EXPERIMENTAL DETAILS**

Present investigation on “Morphological, biochemical and molecular characterization of different genotypes of *Annona* species” was carried out during 2014-15 using Completely Randomized Block Design (CRBD) with three replications.

Leaves, flowers, fruits and seeds were collected from respective genotypes and used as samples for the experimentation.

### **3.5 MORPHOLOGICAL CHARACTERIZATION**

The experimental material was consisted of nine fruiting genotypes raised in Completely Randomized Block Design (CRBD). Three competitive plants were selected randomly from each entry in each replication for recording the observations. The techniques for recording the observations for various leaf, flower, fruit and seed characteristics are described below;

#### **3.5.1 Leaf morphology**

*Annona* possess wide range of leaf dimensions. In this study quantitative as well as qualitative characters related to leaf were measured using following methodologies;

**3.5.1.1 Leaf length (cm):-** Randomly three leaves were selected from each replication of every genotype and length of the leaf was recorded by using Vernier caliper and expressed in centimeters (cm).

**3.5.1.2 Leaf width (cm):-** Randomly three leaves were selected from each replication of every genotype and width of the leaf (middle area) was recorded by using Vernier caliper and expressed in centimeters (cm).

**3.5.1.3 Length of petiole:-** Randomly three leaves were selected from each replication of every genotype and length of petiole was recorded by using Vernier caliper and expressed in centimeters (cm).

**3.5.1.4 Leaf shape:-** Randomly three leaves were selected from each replication of every genotype and types of leaf shape was recorded visually based on leaf margin.

**3.5.1.5 Leaf colour:-** Randomly three leaves were selected from each replication of every genotype and colour of leaf was recorded visually based on the type and intensity of colour.

### **3.5.2 Flower morphology**

**3.5.2.1 Length of the petal (cm):-** Randomly three flowers were selected from each replication of every genotype and length of the petal was recorded by using Vernier caliper and expressed in centimeters (cm).

**3.5.2.2 Width of the petal (cm):-** Randomly three flowers were selected from each replication of every genotype and width of the petal (middle portion) was recorded by using Vernier caliper and expressed in centimeters (cm).

**3.5.2.3 Length of the pedicle (cm):-** Randomly three flowers were selected from each replication of every genotype and length of the pedicle was recorded by using Vernier caliper and expressed in centimeters (cm).

**3.5.2.4 Colour of the flower:-** Randomly three flowers were selected from each replication of every genotype and colour of petal was recorded visually based on type and intensity of the colour during pollination stage.

**3.5.2.5 Shape of the petal:-** Randomly three flowers were selected from each replication of every genotype and shape of petal was recorded on visual basis during pollination stage.

### **3.5.3 Fruit morphology**

**3.5.3.1 Fruit Length (cm):-** Randomly three fruits were selected at the time of ripening stage from each replication of every genotype and length of the fruit was recorded by using Vernier caliper and expressed in centimeters (cm).

**3.5.3.2 Fruit width (cm):-** Randomly three fruits were selected at the time of ripening stage from each replication of every genotype and width of the fruit was recorded by using Vernier caliper and expressed in centimeters (cm).

**3.5.3.3 Fruit weight (g):-** Randomly three fruits were selected at the time of ripening stage from each replication of every genotype and weight was recorded by using weighing balance and fruit weight was expressed in grams (g).

**3.5.3.4 Fruit volume (cm<sup>3</sup>):-** Volume of three randomly selected fruits for every replication was recorded by water displacement method. The fruit was placed in a measuring glass cylinder filled with water and the replaced water was measured for each genotype. Fruit volume was recorded in centimeter cube (cm<sup>3</sup>).

**3.5.3.5 Density of the fruit (g/cm<sup>3</sup>):-** Density of fruits of all the genotypes was calculated by using following formula. Fruit density was measured in grams per centimeter cube (cm<sup>3</sup>).

$$\text{Density of the fruit (g/cm}^3\text{)} = \frac{\text{Weight of the fruit (g)}}{\text{Volume of the fruit (cm}^3\text{)}}$$

**3.5.3.6 Fruit colour:-** Randomly three fruits were selected at the time of ripening stage from each replication of every genotype and colour was recorded visually based on type and intensity of the colour of rind of the fruits.

**3.5.3.7 Fruit shape:-** Randomly three fruits were selected at the time of ripening stage from each replication of every genotype and shape was recorded visually on the basis of shape of the rind of the fruits.

**3.5.3.8 Number of fruits per plant:-** Randomly three plants were selected at the time of ripening stage from each replication of every genotype and total number of fruits per plant were counted.

**3.5.3.9 Fruit yield (kg/plant):-** Total number of fruits per plant was counted for each replication of every genotype and average weight of fruits per plant was calculated. Fruit yield was expressed as kilograms per plant (kg/Plant).

#### **3.5.4 Seed morphology**

**3.5.4.1 Seed length (cm):-** Randomly three seeds were selected from the fruits of each replication of every genotype and length of the seed was recorded using Vernier caliper and expressed in centimeters (cm).

**3.5.4.2 Seed width (cm):-** Randomly three seeds were selected from the fruits of each replication of every genotype and width of the seed (middle portion) was recorded using Vernier caliper and expressed in centimeters (cm).

**3.5.4.3 No. of the seeds per fruit:-** Total number of seeds were counted from three randomly selected ripened fruits of each replication of every genotypes.

**3.5.4.4 100 seed weight (g):-** A total of hundred seeds were collected from each replication of every genotype and weight was measured by using weighing balance. The weight was expressed in grams (g).

**3.5.4.5 Seed shape:-** Randomly three seeds were selected from the fruits of each replication of every genotype and shape of the seed coat was recorded on visual basis.

**3.5.4.6 Seed colour:-** Randomly three seeds were selected from the fruits of each genotype and colour was recorded visually based on the type and intensity of colour of seed coat.

### **3.6 BIOCHEMICAL CHARACTERIZATION**

Three fully and freshly ripened fruits of similar size and same ripening stage were collected randomly from each replication of all nine genotypes. Sample was extracted and estimation of various biochemical parameters was done in three replications.

#### **3.6.1 Qualitative / Proximate compositions of different *Annona* genotypes**

Following eleven fruit quality parameters were recorded from pulps and seeds of different genotypes of *Annona* species and comparison of different *Annona* genotypes was done to find out the better genotypes which can be suggested for improvement of the *Annona* crop as well as to the consumers (Table 3.2).

**Table 3.2 List of quality parameters analyzed for different *Annona* genotypes**

| S.No. | Plant part | Parameters                  |
|-------|------------|-----------------------------|
| 1.    | Fruit pulp | (i). Moisture content       |
|       |            | (ii). Ash content           |
|       |            | (iii). Carbohydrate content |
|       |            | (iv). TSS content           |
|       |            | (v). Reducing sugar         |
|       |            | (vi). Protein content       |
|       |            | (vii). Fibre content        |
|       |            | (viii). Phenol content      |
|       |            | (ix). Titratable acidity    |
|       |            | (x). Ascorbic acid          |
| 2.    | Seed       | (xi). Oil content           |

#### 3.6.1.1 Determination of moisture content:

Moisture content of *Annona* fruit pulp was determined by drying the weighed pulp at 105<sup>0</sup>C for 5 hours and the loss of weight was expressed as moisture content (A.O.A.C., 1980). The total moisture content was calculated based on the following formula;

$$\text{Moisture (\%)} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100$$

#### 3.6.1.2 Determination of ash content:

Ash content of *Annona* fruit pulp was determined by igniting weighted pulp at 600°C temperature for 3-4 hours and the inorganic material left at last was expressed as ash content (Sadasivam and Manickam., 1996). The total ash content was calculated as;

$$\text{Ash (\%)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

### 3.6.1.3 Extraction and estimation of total carbohydrates:

Total carbohydrate was extracted by following the method suggested by Dreywood *et al.* (1946) with some modifications. The 0.5 g sample (fruit pulp) was homogenized in 2N hydrochloric acid using mortar and pestle and volume was made to 20 ml using 2N HCl. The content was refluxed for one hour on boiling water bath at 70°C. Supernatant was collected and residue was re-extracted twice with 2N HCl. All supernatant were pooled and final volume was made to 50 ml using 2N HCl. The extract was used for the estimation of total carbohydrates.

Total carbohydrate was estimated by following the method suggested by Dubois *et al* (1956) with some modifications. Suitable aliquot (0.5 ml) was taken and volume was made to 3 ml with the distilled water followed by addition of 0.5 ml distilled phenol and mixed thoroughly. To this, 5.0 ml concentrated sulphuric acid was carefully added at the side of the tube. After the mixing thoroughly the tubes were kept for 30 minutes at room temperature for colour development.

The absorbance was measured at 490 nm. The content was calculated from the reference curve prepared from D-glucose as standard and expressed as percentage.

The total carbohydrate content was calculated based on the following formula;

$$\text{Carbohydrate (\%)} = \text{Graph factor} \times \frac{\text{Sample reading}}{\text{Weight of sample}} \times \frac{\text{Total volume}}{\text{Taken volume}} \times 10^{-4}$$

#### **3.6.1.4 Extraction and estimation of total Soluble Sugars:**

The 0.5 g sample (fruit pulp) was homogenized in 80% ethanol using mortar and pestle and volume was made to 10 ml using 80% ethanol. The content was refluxed for one hour on boiling water bath at 70°C. Supernatant was collected and residue was re-extracted twice with 80% ethanol. All supernatant were combined and final volume was made to 25 ml using 80% ethanol. The extract was used for the assay of total soluble sugars and reducing sugars (3.6.1.5).

Total soluble sugars were estimated by following the method suggested by Dubois *et al.* (1956) with some modifications. Suitable aliquot (0.5 ml) was taken and volume made to 3 ml with the distilled water followed by addition of 0.5 ml distilled phenol and mixed thoroughly. To this, 5.0 ml concentrated sulphuric acid was carefully added at the side of the tube. After the mixing thoroughly the tubes were kept for 30 minutes at room temperature for colour development. The absorbance was measured at 490 nm. The content was calculated with the help of a reference curve prepared from D-glucose as standard and expressed as percentage.

$$\text{TSS (\%)} = \text{Graph factor} \times \frac{\text{Sample reading}}{\text{Weight of sample}} \times \frac{\text{Total volume}}{\text{Taken volume}} \times 10^{-4}$$

#### **3.6.1.5 Estimation of reducing sugars:**

Reducing sugars were extracted as described in the section 3.6.1.4 and the content was estimated by the method suggested by Nelson (1944).

Suitable quantity of aliquot (0.5 ml) was taken and made the final volume 1.0 ml with distilled water. To this 1.0 ml of alkaline copper reagent (4.0 g. copper sulphate, 21.0 g. sodium carbonate, 12.0 g. sodium potassium tartrate, 16.0 g. sodium bicarbonate, 180 g. anhydrous sodium sulphate dissolved in 1000 ml of distilled water) was added and the tubes were incubated in boiling water bath for 10 minutes. The content was cooled and 1.0 ml of arsenomolybdate reagent (50 g. ammonium molybdate, 42 ml concentrated sulphuric acid, 6.0 g. disodium hydrogen arsenate in distilled water made up 1000 ml) was added to each tube. Finally total volume was made up to 10 ml with distilled water. The absorbance was measured at 540 nm. Reducing sugar content was calculated from the standard curve prepared from D-glucose using following formula;

$$\text{Reducing sugar (\%)} = \text{Graph factor} \times \frac{\text{Sample reading}}{\text{Weight of sample}} \times \frac{\text{Total volume}}{\text{Taken volume}} \times 10^{-4}$$

### 3.6.1.6 Extraction and estimation of proteins:

Protein content was determined by method developed by Lowry *et al.* (1951).

One gram sample (fruit pulp) was weighed and homogenized in 5 ml 0.1 N NaOH and filtered through Whatman No.1 filter paper. The sample extracts (2.5 ml) was taken and made to 3.0 ml volume with distilled water. 5 ml of alkaline copper reagent C was added. Reagent C was prepared by mixing 50 ml of reagent A (2% sodium carbonate in 0.1N sodium hydroxide) with 1.0 ml reagent B (0.5 % copper sulphate in 10 % sodium potassium tartrate).

This reagent was prepared fresh at the time of use and mixed well. The content was allowed to stand for 10 minutes at room temperature followed by addition of 0.5 ml Folin-Ciocalteu Reagent (diluted with water 1:1 v/v). The content was kept for 30 minutes at room temperature and the absorbance was measured at 750 nm. The protein content was calculated using bovine serum albumin (BSA) as standard.

$$\text{Protein (\%)} = \text{Graph factor} \times \frac{\text{Sample reading}}{\text{Weight of sample}} \times \frac{\text{Total volume}}{\text{Taken volume}} \times 10^{-4}$$

### 3.6.1.7 Estimation of fibre content:

Fibre content was determined by the method given by Maynard (1970).

Fresh 1.0 g sample (fruit pulp) was taken and extracted with petroleum ether to remove fat. After extraction, 200 ml sulphuric acid added into dried sample for 30 minute and then sample was filter. After this, the sample was boiled with 200 ml of NaOH for 30 min. and again filtered the sample and washed with solution of 25 ml of boiling sulphuric acid, 50 ml water and 25 ml alcohol. Then the residue was removed and transfered into the pre-weighted dish W1 and dried the residue for 2 h at 130° C (W2). After this the sample was ignited at 130° C for 30 minutes and then cooled to room temperature and reweighted (W3).

$$\text{Crude fibre (\%)} = \frac{\text{Loss of weight on ignition (W2-W1) - (W3-W1)}}{\text{Weight of sample}} \times 100$$

### 3.6.1.8 Estimation of phenol content:

Total phenol was estimated by following the method of Bray and Thorpe (1954) with some modifications.

The 0.5 g sample (fruit pulp) was homogenized in 80% ethanol using mortar and pestle and volume was made to 10 ml using 80% ethanol. The homogenate was centrifuged at 10,000 rpm for 15 min. Supernatant was collected and residue was re-extracted twice and all supernatants were pooled. The supernatant was used for the assay of phenols.

Suitable quantity of aliquot (0.5 ml) was taken and made the final volume 1.0 ml with distilled water. 0.5ml of Folin – Ciocalteu Reagent was added and after 3 min 20% Na<sub>2</sub>CO<sub>3</sub> was added and the tubes were incubated in boiling water bath for 1 min. and cooled it, and total volume was made upto 10 ml with distilled water. The absorbance was measured at 650 nm. Phenol content was calculated from the standard curve prepared from catechol.

$$\text{Phenol content (\%)} = \text{Graph factor} \times \frac{\text{Total volume}}{\text{Taken volume}} \times \frac{1}{\text{Weight of sample}} \times 10^{-4}$$

### 3.6.1.9 Estimation of titratable acidity:

One gram of sample was extracted with distilled water and filtered with Whatman filter paper-1. Final volume was made to 20 ml using distilled water. Suitable quantity (5 ml) of this aliquot was taken in conical flask and few drops of Phenolphthalein indicator (Phenolphthalein indicator- 0.5% in 80% ethanol) was added and shaken well. Burette was filled with 0.1 N NaOH solution after washing

and rinsing. Titration was done and end point was appearance of pink colour and its persistence for at least for few seconds. Titratable acidity was calculated by the following formula;

$$\text{Titrateable acidity (\%)} = \frac{d \times 0.064 (0.1N \text{ NaOH}) \times c}{a \times b} \times 10^{-4}$$

Where, a = weight of the sample

b = volume of the aliquot taken

c = volume made with distilled water

d = average burette reading.

#### 3.6.1.10 Estimation of seed oil content:

Total oil content from *Annona* seeds was determined by Soxhlet extraction method using petroleum ether (40-50°C) developed by Mehta and Lodha (1993).

5g of *Annona* seeds were extracted for 8 hrs with petroleum ether at 60 – 80 °C (A.O.A.C., 1965). The solvent was distilled out and the flasks were then transferred to oven maintained at 80 °C for 24 h. The flasks were removed from oven and kept in desiccator until it comes to room temperature. The flasks were weighed and per cent oil was calculated as,

$$\text{Oil (\%)} = \frac{\text{Weight of flask + Oil} - \text{Weight of flask}}{\text{Weight of sample (g)}} \times 100$$

#### 3.6.1.11 Extraction and estimation of ascorbic acid content:-

Ascorbic acid was extracted from *Annona* fruit pulp (1 g) by adding 5 ml of meta-phosphoric acid – acetic acid solution (15g meta-phosphoric acid dissolved in 40 ml of glacial acetic acid and the volume made upto 200ml with distilled water) in

a mortar and pestle (Malick and Singh, 1980). A homogenized solution was filtered through Whatman no. 1 filter paper and the filtrate was used as source of ascorbic acid.

The known volume of extracted material was titrated against the indophenol dye (50 mg 2, 6 dichlorophenol indophenol dissolved in 50 ml distilled water and 42 mg sodium bicarbonate was dissolved in it and making final volume of dye upto 200ml). The standard ascorbic acid solution was prepared by accurately weighed 50 mg of ascorbic acid as dissolved in distilled water and volume was made to 50 ml. The known volume of ascorbic acid standard solution was transferred into Erlenmeyer flask containing 5.0 ml extracting solution and titrated rapidly with indophenol solution from 50 ml burette until light pink colour persists. Blank was also run with meta- phosphoric acid solution without the sample. Subtracting blank from standard titration, calculated and expressed concentration of indophenol solution as mg ascorbic acid equivalent to 1.0 ml reagent.

$$\text{Ascorbic acid (mg/100gm)} = \text{dye factor} \times \frac{\text{Total volume}}{\text{Taken volume}} \times \frac{1}{\text{Weight of sample}} \times 10^{-4}$$

### 3.6.2 Screening of antioxidant activities

One gram of sample was extracted in 10 ml of 60% aqueous methanol containing 0.1% HCL. It was shaken for 4 hours at room temperature and centrifuged at 10,000 rpm for 15 min at 10 °C. The solution was filtered and stored in freeze. 1ml of extract was mixed with 3ml FRAP reagent (10 mM TPTZ in 40 mM HCl, 20 mM FeCl<sub>2</sub>.6H<sub>2</sub>O and 0.3 M acetate buffer with pH-3.6) and

incubated it at 10 minute at room temperature. The absorbance was read at 593nm and antioxidant activity was calculated as;

$$\text{Antioxidant activity (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

### 3.6.3 Determination of various phytochemicals

Samples were sun-dried, pulverized and passed through a sieve (about 0.5 mm pore size) to obtain fine powder. Aqueous extract of the sample was prepared by soaking 100 g of the powdered sample in 200 ml of distilled water for 12hrs. The extracts were filtered using whatman filter paper No 42. The following phytochemicals screening was done with methods of Vagashiya *et al.* (2011).

**3.6.3.1 Test for steroid:-** 2 ml of acetic anhydride was added to 0.5 g ethenolic extract of the sample with 2 ml H<sub>2</sub>SO<sub>4</sub>. The colour changed from violet to blue or green in some samples indicating the presences of steroids.

**3.6.3.2 Test for flavenoids:-** The presence of flavenoids in the plant sample was determined by the methods described by Harborne (1973) and Sofowara (1993). 5 ml of dilute ammonia solution (1:1) was added to portion of the aqueous filtrate of the plant extract followed by addition of concentrated H<sub>2</sub>SO<sub>4</sub>. Yellow coloration observed in each extract indicated the presence of flavenoids.

**3.6.3.3 Test for tannins-** About 0.5 g of dried powdered sample was boiled in 20ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

**3.6.3.4 Test for terpenoids:-** (Salkowski test): 5 ml of extract was mixed in 2 ml of chloroform, and 3 ml concentrated  $\text{H}_2\text{SO}_4$  was carefully added to form a layer. A reddish brown coloration of the interface was formed to show positive results for the presences of terpenoids.

**3.6.3.5 Test for phenols:-** 1 ml of extract was dissolved in 0.5 ml of 20%  $\text{H}_2\text{SO}_4$  solution. Followed by addition of few drops of aqueous sodium hydroxide solution, it turns blue which indicates the presence of phenols.

**3.6.3.6 Test for alkaloids:-** (Wagner's test) few drops of Wagner's reagent was added to 5 ml of filtrate by side of the test tube, A reddish brown precipitate confirms the tests as positive.

**3.6.3.7 Test for saponins:-** 5 ml of extract was added to 20 ml of distilled water and agitated well in graduated cylinder for 15 minutes. Formation of foam indicates the presence of saponins.

**3.6.3.8 Test for glycosides:-** 1 ml of water and few drops of aqueous NaOH was added to 0.5mg of extract. Appearance of yellow colour indicated the presence of glycosides.

### 3.7. MOLECULAR CHARACTERIZATION

The molecular diversity analysis of twenty *Annona* genotypes was carried out using molecular markers. It included;

- ❖ Genomic DNA isolation
- ❖ Qualitative and quantitative analysis of genomic DNA
- ❖ Random Amplification of Polymorphic DNA (RAPD)
- ❖ Simple Sequence Repeat (SSR)

#### 3.7.1 Genomic DNA isolation

The genomic DNA was isolated by following Cetyl Trimethyl Ammonium Bromide (CTAB) method given by Doyle and Doyle (1990), with some minor modifications. The chemicals required for DNA isolation are as follows.

##### 3.7.1.1 Preparation of stock solutions

The following stock solutions were prepared well in advance and were used for extracting DNA from the leaves of all twenty *Annona* genotypes.

##### 3.7.1.1.1 1M Tris (pH-8.0)

| Reagent         | Quantity |
|-----------------|----------|
| Tris            | 12.11g   |
| Distilled water | 80ml     |

The solution was adjusted to pH 8.0 with NaOH pellets and volume was made upto 100ml by adding distilled water then solution was stored in a reagent bottle and sterilized by autoclaving.

**3.7.1.1.2 5M NaCl**

| Reagent         | Quantity |
|-----------------|----------|
| NaCl            | 29.92g   |
| Distilled water | 50ml     |

When the salts get completely dissolved, final volume was adjusted to 100ml by distilled water then solution was stored in a reagent bottle and sterilized by autoclaving.

**3.7.1.1.3 0.5M EDTA**

| Reagent         | Quantity |
|-----------------|----------|
| EDTA            | 7.30g    |
| Distilled water | 80ml     |

The solution was adjusted to pH 8.0 with NaOH pellets and volume was made upto 100ml by adding distilled water then solution was stored in a reagent bottle and sterilized by autoclaving.

**3.7.1.1.4 CTAB Extraction Buffer (4%)**

| Stock solution       | Amount per 10 ml |
|----------------------|------------------|
| CTAB                 | 0.4g             |
| 5M NaCl              | 2.8ml            |
| 1M Tris HCl (pH 8.0) | 1.0ml            |
| 0.5 EDTA (pH 8.0)    | 1.0ml            |
| PVP (4%)             | 0.1g             |
| Distilled water      | 4.7ml            |
| <b>Total</b>         | <b>10.0ml</b>    |

1ml of 1M Tris HCl (pH 8.0), 2.8ml of 5M NaCl and 1ml of 0.5M EDTA (pH 8.0) was measured and mixed with about 4ml of hot distilled water, then 0.4g (w/v) CTAB and 0.1g (w/v) PVP was added to this solution. It was stored in a reagent bottle. Just before use 100 $\mu$ l (1%)  $\beta$ -mercaptoethanol was added.

#### 3.7.1.1.5 3.0M Sodium Acetate (pH 5.2)

| Reagent         | Quantity |
|-----------------|----------|
| Sodium Acetate  | 40.81g   |
| Distilled water | 80ml     |

The solution was adjusted to pH 5.2 with NaOH pellets and volume was made upto 100ml by adding distilled water then autoclaved before use.

#### 3.7.1.1.6 Chloroform-Isoamyl alcohol (24:1)

| Reagent         | Quantity |
|-----------------|----------|
| Chloroform      | 96ml     |
| Isoamyl alcohol | 4.0ml    |

It was mixed well and stored in a reagent bottle at room temperature.

#### 3.7.1.1.7 70% Ethanol

| Reagent          | Quantity |
|------------------|----------|
| Absolute ethanol | 70ml     |
| Distilled water  | 30ml     |

The solution was stored in a reagent bottle at 4°C temperature.

### 3.7.1.1.8 100ml, 0.1mM TE Buffer (10mM Tris HCl, 0.1mM EDTA, pH 8.0)

| Reagent         | Quantity |
|-----------------|----------|
| 1M Tris         | 1ml      |
| 0.5M EDTA       | 200µl    |
| Distilled water | 99ml     |

It was mixed thoroughly in a reagent bottle, autoclaved and stored at room temperature.

### 3.7.1.1.9 10mg/ml RNase

100mg of RNase was weighed and dissolved in 0.01M sodium acetate (pH 5.2). The mixture was heated to 100°C for 15 min and allowed to cool to room temperature. The pH 7.4 was adjusted by adding 1M Tris. RNase was stored at -20°C in aliquot.

### 3.7.1.1.10 5X TBE Buffer

| Reagent          | Quantity |
|------------------|----------|
| Tris base        | 54.0g    |
| Boric acid       | 27.5g    |
| 0.5M EDTA (pH-8) | 20.0ml   |
| Distilled water  | 450ml    |

Dissolve the salts and adjust the volume up to 1L with distilled water and pH to 8.0 with 1N NaOH.

### 3.7.1.1.11 Gel loading dye (6X)

For preparing gel loading dye 6X, 0.25% bromophenol blue (BPB), 0.25% of xylene cyanol FF and 30% of glycerol were added to distilled water.

### 3.7.1.1.12 Ethidium Bromide (EtBr) solution (10mg/L, 10ml)

| Reagent          | Quantity |
|------------------|----------|
| Ethidium Bromide | 0.1g     |
| Distilled water  | 10ml     |

It was stirred on a magnetic stirrer for some time to ensure that the dye has dissolved properly. It was stored at 4°C in an amber coloured reagent bottle or wrapped in aluminium foil.

### 3.7.1.2 Genomic DNA isolation (CTAB method)

(i) 300mg of leaf tissue was ground in liquid Nitrogen (N<sub>2</sub>) with the help of mortar & pestle and fine powder so obtained was transferred to 2ml centrifuge tube.

(ii) 1ml pre-warmed (65°C) DNA isolation buffer (CTAB buffer) containing 1% (v/v) β- mercaptoethanol and 1μL of fresh proteinase-K was added to each centrifuge tube and vortexed gently to mix it thoroughly.

(iii) These centrifuge tubes were incubated in hot dry bath at 45°C for 15 min then kept in water bath for 1 hour at 65°C (frequent gentle swirling was done after every 10 min).

(iv) All tubes are cooled at room temperature and equal volume of Chloroform: Isoamylalcohol (24:1) was added and mixed thoroughly.

(v) These tubes were centrifuged at 10,000 rpm for 10 min at 4°C temperature.

(vi) One ml of aqueous phase was transferred to fresh tube and equal volume of Chloroform: Isoamylalcohol (24:1) was added and mixed to ensure emulsification of the phase and again centrifuged at 10,000 rpm for 10 min at 4°C temperature. Step (vi) was repeated 2-3 times, if necessary.

(vii) Aqueous phase was transferred to another fresh tube and equal volume of chilled isopropanol was added to aqueous phase and incubated for 45 min to precipitate DNA.

(viii) DNA-CTAB complex was precipitated as a fibrous network. It was centrifuged at 12,000 rpm for 10 minutes at 4°C temperature.

(ix) After removing supernatant, 200 µl of 70% alcohol was added in pellet and then centrifuged at 10,000 rpm for 5 minutes at 4°C temperature. This step was repeated twice.

(x) The tubes were inverted and drained on a paper towel. The pellet was air dried for 30 minutes.

(xi) Each pellet was dissolved in 100µL of TE buffer by keeping 30 minutes at room temperature with gentle tapping.

(xii) This DNA stock was stored at -20°C temperature.

### **3.7.2 Qualitative and quantitative analysis of genomic DNA**

The quality and quantity (in terms of protein and RNA contamination) of isolated genomic DNA was estimated by spectrophotometer instrument (NanoQuant, Infinite 200). 1µL DNA was loaded to each well and concentration of DNA and absorbance at 260 nm and 280 nm were measured.

To check the form of DNA (linear or sheared) and RNA contamination of isolated genomic DNA, electrophoresis was done using 0.8% agarose gel and quality was judged by viewing the image of single compact DNA band.

#### **3.7.2.1 DNA dilution**

50ng/µl of working DNA solution was prepared from the known quantity of stock DNA solution by using formula  $N_1V_1 = N_2V_2$ . Where  $N_1$  was concentration of DNA stock,  $V_1$  was volume of stock to be taken for dilution,  $N_2$  was concentration of working DNA solution (50ng) and  $V_2$  was volume of working DNA solution.

#### **3.7.2.2 Primer dilution**

10pmol (Pico mole) of primer (working solution) was prepared from the known quantity of primer (stock solution) by using formula  $N_1V_1 = N_2V_2$ . Where  $N_1$  was concentration of primer stock (100pmol),  $V_1$  was volume of stock to be taken for dilution,  $N_2$  was concentration of working primer and  $V_2$  was volume of working primer.

### 3.7.3 Random Amplified Polymorphic DNA (RAPD) analysis

For RAPD analysis, genomic DNA was amplified using random primers Operon Tech., California, USA. Random primers belonged to OPA, OPB, OPC, OPD, OPE and OPD series were used for preliminary screening, of which following eleven primers showed maximum amplification in all 20 *Annona* genotypes (Table 3.3).

**Table-3.3 List of RAPD Primers used in molecular analysis of *Annona* genotypes**

| S.No. | Primer series | Primer sequence (5'-3') |
|-------|---------------|-------------------------|
| 1.    | OPB-6         | TGCTCTGCCC              |
| 2.    | OPB-7         | GGTGACGCAG              |
| 3.    | OPB-8         | GTCCACACGG              |
| 4.    | OPB-10        | CTGCTGGGAC              |
| 5.    | OPB-12        | CCTTGACGCA              |
| 6.    | OPD-2         | GGACCCAACC              |
| 7.    | OPD-16        | AGGGCGTAAG              |
| 8.    | OPD-20        | ACCCGGTCAC              |
| 9.    | OPF-4         | GGTGATCAGG              |
| 10.   | OPF-5         | CCGAATTCCC              |
| 11.   | OPF-10        | GGAAGCTTGG              |

#### 3.7.3.1 PCR reaction mixture for RAPD

A reaction of 15µl volume was prepared for amplification of DNA. The PCR reaction mixture contained the following components;

| S.No.        | Reagents for PCR reaction mixture                               | Volume (µl) |
|--------------|-----------------------------------------------------------------|-------------|
| 1.           | PCR Dream taq buffer with MgCl <sub>2</sub> (Thermo scientific) | 2.00        |
| 2.           | dNTPs (Thermo scientific)                                       | 0.40        |
| 3.           | Dream <i>Taq</i> polymerase (5U/µl, Thermo scientific)          | 0.20        |
| 4.           | Primer (10 pmol)                                                | 1.00        |
| 5.           | Template DNA (50 ng)                                            | 1.20        |
| 6.           | Nuclease free water                                             | 10.20       |
| <b>Total</b> |                                                                 | <b>15.0</b> |

### 3.7.3.2 PCR conditions for RAPD assay

It was allowed for amplification in polymerase chain reaction (PCR) machine called thermo cycler (Eppendorf Mastercycler ep model no. 5341). The following steps were set in thermo cycler;

| S.No. | Steps                | Temperature (°C) | Duration |
|-------|----------------------|------------------|----------|
| 1.    | Initial denaturation | 94               | 7 min    |
| 2.    | Denaturation         | 94               | 50 sec   |
| 3.    | Annealing            | 38               | 50 sec   |
| 4.    | Extension            | 72               | 1.2 min  |
| 5.    | Final extension      | 72               | 7 min    |
| 6.    | Hold                 | 4                | 10 min   |

### 3.7.4 Simple Sequence Repeats (SSR)

In this experiment, the genomic DNA was also amplified using SSR primers (Eurofins MWG Operon). A set of total twenty microsatellite primers (Escribano *et al.*, 2008) were selected for preliminary screening, of which twelve primers which



showed polymorphism were utilized for studying the genetic diversity among twenty *Annona* genotypes (Table 3.4).

#### 3.7.4.1 PCR reaction mixture for SSR

The PCR reaction mixture of 10µl volume was prepared in 200µl thin walled PCR tubes (Eppendorf Master cycler ep model no. 5341) for cyclic amplification. The PCR reaction mixture contained the following components;

| S.No.        | Reagents                                                        | Volume (µl) |
|--------------|-----------------------------------------------------------------|-------------|
| 1.           | PCR Dream Taq buffer with MgCl <sub>2</sub> (Thermo scientific) | 1.00        |
| 2.           | dNTPs (Thermo scientific)                                       | 0.30        |
| 3.           | Dream <i>Taq</i> polymerase (5U/µl) (Thermo scientific)         | 0.15        |
| 4.           | Forward Primer (Eurofins MWG Operon)                            | 0.30        |
| 5.           | Reverse primer (Eurofins MWG Operon)                            | 0.30        |
| 6.           | Template DNA (50 ng)                                            | 1.00        |
| 7.           | Nuclease free water                                             | 6.85        |
| <b>Total</b> |                                                                 | <b>10.0</b> |

#### 3.7.4.2 PCR conditions for SSR assay

The PCR tubes containing PCR reaction mixture were gently shaken for proper mixing up of ingredients. These PCR tubes were allowed to keep in thermal cycler machine and following conditions or steps were set in thermo cycler for primer amplification;

| S.No. | Steps                | Temperature (°C) | Duration |
|-------|----------------------|------------------|----------|
| 1.    | Initial denaturation | 94               | 7 min    |
| 2.    | Denaturation         | 94               | 50 sec   |
| 3.    | Annealing            | $\Delta T_m$     | 50 sec   |
| 4.    | Extension            | 72               | 1.0 min  |
| 5.    | Final extension      | 72               | 7 min    |
| 6.    | Hold                 | 4                | 10 min   |

\* $T_m$  were selected according to the GC content of respected primers

### 3.7.5 Electrophoresis of RAPD and SSR amplified products

Agarose gel (Amresco, Solan Industry, Solan) of 1.5% for and 2.5 % concentration for RAPD and SSR, respectively, was prepared in 0.5 X TBE buffer. 3.0  $\mu$ l of Ethidium Bromide (Amresco, USA) working stock was added to it. PCR amplified products of RAPD and SSR were loaded into the wells separately. 2 $\mu$ l of 100+ bp standard ladder (Thermo scientific, USA) was also run along with the samples. Electrophoresis was conducted at 120 V current (constant) to separate the amplified bands. The separated bands were visualized under UV transilluminator and photographed using gel documentation system.

## 3.8 STATISTICAL ANALYSIS

### 3.8.1 Analysis of morphological and biochemical data

In case of morphological and biochemical parameters, the data obtained during the investigation were subjected to the statistical analysis. The method of analysis for RBD was used in the experiment.

The appropriate standard error of mean (S.Em), CD at 5% level of probability were calculated in each case. Coefficient of Variance (CV) was also calculated for judging the significance. Correlation coefficient was estimated for each parameter to find out the simple correlation between different parameters. Heritability, Genetic advance, GCV and PCV were also calculated.

### 3.8.1.1 Analysis of Variance

Data recorded for all the characters were subjected to the analysis of variance with the formula suggested by Panse and Sukhatme (1967). The following statistical model for randomized block design was used;

$$Y_{ij} = \mu + \beta_i + \tau_j + \varepsilon_{ij}$$

Where,  $Y_{ij}$  = Mean phenotypic value of  $j^{\text{th}}$  genotype in  $i^{\text{th}}$  replication

$\mu$  = General mean

$\beta_i$  = Effect due to  $i^{\text{th}}$  replication

$\tau_j$  = Effect due to  $j^{\text{th}}$  genotype

$\varepsilon_{ij}$  = Uncontrolled variation associated with  $j^{\text{th}}$  genotype in  $i^{\text{th}}$  replication

#### Analysis of variance for RCBD

| Source       | Degree of freedom | Mean Square | Expected Mean Square        |
|--------------|-------------------|-------------|-----------------------------|
| Replications | (r-1)             | $M_r$       | $\sigma_e^2 + g \sigma_r^2$ |
| Genotypes    | (g-1)             | $M_g$       | $\sigma_e^2 + r \sigma_g^2$ |
| Error        | (r-1) (g-1)       | $M_e$       | $\sigma_e^2$                |
| Total        | (rt-1)            | -           | -                           |

Where, r = number of replications

g = number of genotypes

### 3.8.1.2 Test of Significance

Test of significance for various mean square was carried out by ‘F’ test. The ‘F’ values were calculated as under;

$$\text{Genotypes} = \frac{M_g}{M_e}$$

### 3.8.1.3 Critical difference of the estimates

To test the significance of differences of the estimates, critical difference was calculated as:

$$\text{S. Ed.} = \sqrt{\frac{2M_e}{r}}$$

$$\text{C. D.} = \text{S. Ed.} \times t$$

Where, t = Table ‘t’ value for error degree of freedom at 0.05 and 0.01 levels of probability.

### 3.8.1.4 Co-efficient of variation (C.V. %)

The co-efficient of variation for each character was calculated as under,

$$\text{C.V.}\% = \frac{\sqrt{M_e}}{\bar{X}} \times 100$$

Where, Me = Error mean square

$\bar{X}$  = General mean for the character.

### 3.8.1.5 Genetic coefficient of variation (GCV %)

It was computed using the formula given by Burton (1952).

$$\text{GCV (\%)} = \frac{\sqrt{\sigma_g^2}}{\bar{X}} \times 100$$

Where,  $\sigma_g^2$  = Genotypic variance

$\bar{X}$  = General mean of character under study.

### 3.8.1.6 Phenotypic coefficient of variation (PCV %)

It was computed using the formula given by Burton (1952).

$$\text{PCV (\%)} = \frac{\sqrt{\sigma_p^2}}{\bar{X}} \times 100$$

Where,  $\sigma_p^2$  = Phenotypic variance

$\bar{X}$  = General mean of character under study.

Genotypic coefficient of variance (GCV) and phenotypic coefficient of variance (PCV) were classified as suggested by Shivasubramanian and Menon (1973) as follows;

0-10% = Low

10-20% = Moderate

20% and above = High

### 3.8.1.7 Heritability in broad sense ( $H^2$ )

It is the proportion of phenotypic variability that is due to genetic reasons. It was computed in percentage using following formula given by Allard (1960).

$$H^2 b (\%) = \frac{\sigma_g^2}{\sigma_p^2} \times 100$$

Where,  $H^2b$  = Heritability in broad sense

$\sigma_g^2$  = Genotypic variance

$\sigma_p^2$  = Phenotypic variance

Heritability percentage was categorized as,

0-30% = Low

30-60% = Moderate

60% and above = High

### 3.8.1.8 Genetic advance as percent of mean

Expected genetic advance (GA) was calculated for each character by adopting the procedure suggested by Allard (1960);

$$GA = k \times H^2b \times \sigma_p$$

Where,  $H^2b$  = Heritability in broad sense

$K$  = Selection differential at 5% intensity (2.06) of selection

$\sigma_p$  = Phenotypic standard deviation

The expected genetic advance expressed as percentage of mean was estimated as follows;

$$GA (\% \text{ of mean}) = \frac{GA}{\bar{X}} \times 100$$

Where, GA = Expected genetic advance

$\bar{X}$  = Mean value of the character

Genetic advance as percent mean was categorized as suggested by Johnson *et al.*, (1955).

0-10% = Low

10-20% = Moderate

20% and above = High

### **3.8.2 Analysis of molecular data**

#### **3.8.2.1 Data analysis**

For molecular analysis, clear and distinct bands amplified by RAPD and SSR primers were scored for the presence and absence of bands among 20 *Annona* genotypes. The score '1' and '0' indicated the presence and absence of bands, respectively. The data were entered into binary matrix and subsequently analyzed using NTSYSpc version 2.02 (Rohlf,1998).

#### **3.8.2.2 Genetic similarity and cluster analysis**

Coefficients of similarity were calculated by using Jaccard's similarity coefficient by SIMQUAL function and cluster analysis was performed by agglomerative technique using the Un-weighted Pair Group Method with Arithmetic Mean (UPGMA) method by SAHN clustering function of NTSYSpc.

Relationships among the *Annona* genotypes were expressed in the form of dendrograms and genetic similarity matrix. .

### 3.8.2.3 Principal Component Analysis (PCA)

Principal component analysis was carried out using the EIGEN module of NTSYSpc 2.02. The results were graphically expressed in the form of 2D and 3D plots generated by graphics module after the calculation of EIGEN values. First three Eigen values which showed maximum variation were extracted as principal components and 2D and 3D plots were generated on that basis.

### 3.8.2.4 Calculation of parameters of genetic variability

Various components were calculated which included, No. of monomorphic and polymorphic loci, Polymorphism Information Content (PIC), Effective Multiplex ratio (EMR), Marker Index (MI) and Polymorphism percentage.

**(i) Polymorphism Information content (PIC)** was calculated according to formula described by Bootstein *et al.* (1980).

$$PIC = 1 - \sum_{i=1}^k P_i^2$$

where  $k$  is total number of alleles detected for a given marker locus and  $P_i$  is the frequency of the  $i^{\text{th}}$  allele in the set of genotypes investigated.

**(ii) Percentage polymorphism** was calculated as follows;

$$\text{Polymorphism (\%)} = \frac{\text{No. of polymorphic loci}}{\text{Total no. of loci}} \times 100$$

All the above mentioned variables were calculated individually for RAPD and SSR markers to identify genetic diversity among the *Annona* genotypes.

## IV. RESULTS AND DISCUSSION

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The present investigation was undertaken with a view to characterize various genotypes of *Annona* species based on morphological, biochemical and molecular parameters. The results obtained from this study are summarized and discussed below;

### **4.1 Morphological characterization:**

- 4.1.1 Leaf morphology
- 4.1.2 Flower morphology
- 4.1.3 Fruit morphology
- 4.1.4 Seed morphology
- 4.1.5 Genetic variability components for different morphological parameters
- 4.1.6 Correlation analysis of different morphological parameters

### **4.2 Biochemical characterization:**

- 4.2.1 Qualitative parameters/proximate composition
- 4.2.2 Study of antioxidant activity in pulp and seeds
- 4.2.3 Determination of phytochemicals in pulp and seeds
- 4.2.4 Genetic variability components for different biochemical parameters
- 4.2.5 Correlation analysis of different biochemical parameters

### **4.3 Molecular characterization:**

- 4.3.1 Qualitative and quantitative analysis of genomic DNA
- 4.3.2 Random Amplification of Polymorphic DNA (RAPD) analysis

#### 4.3.3 Simple sequence repeats (SSRs) analysis

#### 4.3.4 Comparison of RAPD and SSR markers

### 4.1 MORPHOLOGICAL CHARACTERIZATION

A total of nine genotypes were evaluated for various qualitative and quantitative traits related to leaves, flowers, fruits and seeds. The result is presented as follows;

#### 4.1.1 Leaf morphology

*Annona* contain wide range of leaf length and leaf width with different shapes like oblong, lanceolate to oval. All values presented in Table 4.1 are mean values of different genotypes. The results obtained are briefly summarized as follows (Fig 4.1 and 4.2);

##### 4.1.1.1 Leaf length (cm)

Leaf length of different genotypes of *Annona* was measured and presented in Table 4.1. Leaf length ranged from 5.14 to 21.38 cm. Highest leaf length was recorded for *A. atemoya* (21.38 cm) followed by *A. cherimola* (18.31 cm), *A. reticulata* (15.65 cm) and *A. muricata* (15.05 cm). Lowest leaf length was recorded for Red Sitaphal (5.14 cm). Leaf length for GJCA-1, Sindhan, Balanagar and Anand Selection was 9.13, 9.12, 8.99 and 8.26 cm respectively.

This suggested the significant diversity existed among different *Annona* genotypes for leaf length, which can be utilized for selection in crop improvement programme.







#### 4.1.1.2 Leaf width (cm)

Different genotypes of *Annona* were studied for measurement of leaf width. Leaf width ranged from 2.22 to 10.70 cm (Table 4.1) in different genotypes. Highest leaf width was recorded for *A. atemoya* (10.70 cm) followed by *A. cherimola* (9.36 cm), *A. muricata* (6.06 cm) and *A. reticulata* (4.85 cm). Lowest leaf width was recorded for Red Sitaphal (2.22 cm), which was at par with Anand Selection (2.88 cm). Leaf width for Sindhan, Balanagar and GJCA-1 was 3.03 cm, 3.01 cm and 3.01 cm respectively.

This suggested the significant diversity existed among different *Annona* genotypes for leaf width, which can be utilized for crop improvement programme.

#### 4.1.1.3 Length of petiole (cm)

Length of petiole for different genotypes of *Annona* was measured. Petiole length was varied from 0.71 to 2.20 cm (Table 4.1). Maximum petiole length was recorded for *A. atemoya* (2.20 cm) followed by *A. cherimola* (1.93 cm), *A. reticulata* (1.33 cm), whereas minimum petiole length was recorded for Red Sitaphal (0.71 cm) which was at par with Anand Selection (0.84 cm). Petiole length for Balanagar, GJCA-1, Sindhan and *A. muricata* was 0.93, 0.93, 0.90 and 0.89 cm, respectively.

This suggested that petiole length possessed significant diversity among different *Annona* genotypes which can be utilized for crop improvement programme.

#### **4.1.1.4 Leaf shape**

Leaf shape was ovate to elliptical in *A. cherimola*, oblong lanceolate in *A. reticulata*, ovate oblong to cylindrical in *A. muricata*, ovate to elliptical in *A. atemoya* and ovate to lanceolate in different genotypes of *A. squamosa* (Table 4.4).

#### **4.1.1.5 Leaf colour**

Leaf colour was dull medium green in *A. cherimola*, glossy light green in *A. reticulata*, glossy dark green in *A. muricata*, dull dark green in *A. atemoya* and light to medium green in different genotypes of *A. squamosa* (Table 4.4).

### **4.1.2 Flower morphology**

*Annona* produce flowers with wide range of size, shape, colour and flavor. A brief summary of results obtained in the present investigation is as follows (Fig 4.3 and 4.4);

#### **4.1.2.1 Length of the petal (cm)**

Petal length of flowers of different genotypes of *Annona* was recorded which ranged from 2.35 to 4.35 cm (Table 4.1). Maximum length of petal was recorded for *A. atemoya* (4.35 cm) followed by Anand Selection (3.55 cm), Sindhan (3.45 cm), *A. cherimola* (3.22 cm). Minimum length of petal was recorded for *A. reticulata* (2.35 cm) which was at par with *A. muricata* (2.36 cm). Length of petal for Red Sitaphal, Balanagar and GJCA-1 was 2.72, 2.93 and 2.79cm, respectively. This suggested that petal length possessed significant diversity among different *Annona* genotypes which can be utilized for crop improvement programme.





#### 4.1.2.2 Width of the petal (cm)

Different genotypes of *Annona* species were studied for their petal width and result obtained in the ranged of 0.63 to 1.44 cm (Table 4.1). Maximum width of petal was recorded for *A. muricata* (1.44 cm) followed by *A. atemoya* (1.00 cm), Sindhan (1.00 cm) and Balanagar (0.87 cm). Minimum width of petal was recorded for Red Sitaphal (0.63 cm) which was at par with *A. cherimola* (0.74 cm), *A. reticulata* (0.65 cm), Anand Selection (0.74 cm) and GJCA-1 (0.72 cm).

*Annona* possessed significant diversity for Petiole width, which can be utilized for improvement of the crop.

#### 4.1.2.3 Length of pedicel (cm)

Length of pedicel for different genotypes of *Annona* was in the range of 1.08 to 3.16 cm (Table 4.1). Maximum length of pedicel was recorded for *A. reticulata* (3.16 cm) followed by *A. atemoya* (2.44 cm), Sindhan (2.40 cm) and *A. cherimola* (2.31 cm). Minimum length of pedicel was recorded for *A. reticulata* (1.08 cm). Remaining genotypes like Anand Selection, Balanagar, GJCA-1 and Red Sitaphal possessed pedicel length of 1.96, 1.86, 1.69 and 1.29 cm, respectively.

There was significant diversity among different *Annona* genotypes for pedicel length and this diversity can be utilized for improvement of the crop.

#### 4.1.2.4 Petal colour

Petal colour varied from pale green in *A. cherimola*, yellowish green in *A. reticulata*, creamish yellow in *A. muricata*, medium yellowish green in *A. atemoya*



and light pale green in different genotypes of *A. squamosa* except for Red Sitaphal which possessed pale green with reddish tinge colored petal (Table 4.4).

#### **4.1.2.5 Petal shape**

Oblong shaped petals were observed in *A. cherimola*, *A. reticulata*, *A. atemoya* and *A. squamosa* except for *A. muricata* which possessed ovate shaped petals (Table 4.4).

#### **4.1.3 Fruit morphology**

Fruits are important product of *Annona* trees. This study observed wide variation in the fruit characters like fruit length, fruit width, fruit weight, fruit volume, fruit density and number of fruits per plant (Fig 4.5, 4.6 and 4.7). Results obtained in present investigation are as follows;

##### **4.1.3.1 Fruit length (cm)**

Fruit length for different genotypes of *Annona* was recorded, which was found in the range of 6.87 to 10.95 cm (Table 4.2). Significantly higher length of fruit was recorded for *A. muricata* (10.95 cm) which was at par with *A. atemoya* (10.35 cm), whereas significantly lower length of fruit was recorded for Red Sitaphal (6.87 cm), which was at par with GJCA-1 (7.87 cm) and Anand Selection (7.71 cm). Length of fruit for *A. cherimola*, *A. reticulata*, Balanagar and Sindhan was 9.32, 9.25, 9.06 and 8.44 cm, respectively.







This wide range of diversity can be used for identifying and selecting the genotypes of desirable length thus improvement of the crop through breeding methods.

#### **4.1.3.2 Fruit width (cm)**

Different genotypes of *Annona* were studied for fruit width and results were obtained in the range of 6.72 to 10.37 cm (Table 4.2). Significantly higher fruit width was recorded for *A. atemoya* (10.37 cm) followed by *A. cherimola* (9.29 cm), Balanagar (9.19 cm), Sindhan (8.52 cm), *A. reticulata* (8.29 cm), GJCA-1 (8.23 cm) and Anand Selection (7.71 cm), while Red Sitaphal showed significantly lower fruit width (6.72 cm), which was at par with *A. muricata* (7.71 cm).

Above result suggested the presence of wide range of fruit width and this diversity can be useful in selecting genotypes with large sized fruits.

#### **4.1.3.3 Fruit weight (g)**

Fruit weight was observed in the range of 87.89 to 370.41 g for different genotypes of *Annona* (Table 4.2). Significantly higher fruit weight was recorded for *A. atemoya* (370.41 g) followed by *A. muricata* (281 g), *A. cherimola* (225.39 g) and *A. reticulata* (221 g), whereas significantly lower fruit weight was recorded for Red Sitaphal (87.89 g). Fruit weight for Balanagar, Sindhan, GJCA-1 and Anand Selection was found 177.59, 163.47, 142.24 and 136.02 g, respectively (Fig 4.10).

High diversity present among *Annona* genotypes provides opportunity for selecting genotypes with higher fruit weight and can be used for improvement of the fruit yield.

#### **4.1.3.4 Fruit volume (cm<sup>3</sup>)**

Volume of fruits for different genotypes of *Annona* was measured. Fruit volume was found in the range of 92 to 340 cm<sup>3</sup> (Table 4.2). *A. atemoya* showed significantly higher volume of fruit (340.00 cm<sup>3</sup>) which was followed by *A. muricata* (249.17 cm<sup>3</sup>), *A. cherimola* (206.67 cm<sup>3</sup>) and *A. reticulata* (195.67 cm<sup>3</sup>) and significantly lower fruit volume was recorded for Red Sitaphal (92 cm<sup>3</sup>). Fruit volume for Balanagar, Sindhan, Anand Selection and GJCA-1 was found 186.67, 157, 137.97 and 137.67 cm<sup>3</sup>, respectively.

Fruit volume is an important character for consumer's choice and genotypes with higher fruit volume can be selected for developing higher yielding *Annona* cultivars.

#### **4.1.3.5 Fruit density (g/cm<sup>3</sup>)**

Fruit density of different genotypes of *Annona* was measured and presented in Table 4.2. Fruit density was found in the range of 0.96 to 1.13 g/cm<sup>3</sup>. Maximum density of fruit was recorded for *A. muricata* (1.13 g/cm<sup>3</sup>) followed by *A. cherimola* (1.08 g/cm<sup>3</sup>), *A. atemoya* (1.08 g/cm<sup>3</sup>), and *A. reticulata* (1.06 g/cm<sup>3</sup>), whereas minimum density of fruit was recorded for Red Sitaphal (0.96 g/cm<sup>3</sup>). Fruit density



for Sindhan, GJCA-1, Anand Selection and Balanagar was found 1.04, 1.03, 1.0 and 0.99 g/cm<sup>3</sup>, respectively.

Higher fruit density indicates the presence of more amounts of biochemical compounds in the limited volume of fruit. For the improvement of this fruit quality, genotypes with higher fruit density can be selected and utilized in through breeding programmes.

#### **4.1.3.6 Number of fruits per plant**

Average number of fruits per plant for different genotypes of *Annona* was recorded in the range of 53.33 to 116.33 (Table 4.2). Significantly higher number of fruits per plant was recorded for *A. cherimola* (116.33) which was at par with Sindhan (110) and GJCA-1(107), whereas significantly lower number of fruits per plant was recorded for *A. muricata* (53.33) which was at par with *A. atemoya* (57). Number of fruits per plant for Balanagar, Anand Selection, *A. reticulata* and Red Sitaphal was 98.67, 95, 90.33 and 85.67, respectively (Fig 4.10).

Above result suggested that *Annona* genotypes possessed higher diversity for number of fruits per plant which is an important yield attributing trait. Genotypes with higher no. of fruits per plant can be selected and utilized in improvement of crop for this character.

#### **4.1.3.7 Fruit yield per plant (kg/plant)**

Fruit yield per plant for different genotypes of *Annona* was measured and presented in Table 4.2. Fruit yield found in range of 7.53 to 26.47 kg/plant.

Significantly higher fruit yield per plant was recorded for *A. cherimola* (26.47 kg/plant), whereas Red Sitaphal showed significantly lower yield per plant (7.53 kg/plant). Fruit yield for *A. atemoya*, *A. reticulata*, Balanagar, Sindhan, GJCA-1, *A. muricata* and Anand Selection was found to be 21.83, 19.50, 17.67, 17.62, 15.09, 15.01 and 12.87 kg per plant, respectively (Fig 4.10)

Higher fruit yield per plant is the ultimate goal of the breeders and producers. Crop improvement program can be taken up by selecting genotypes with higher fruit yield.

#### **4.1.3.8 Fruit colour (rind)**

Fruit colour varied from light yellowish green in *A. cherimola*, yellowish green with reddish tinge in *A. reticulata*, shiny dark green to light green in *A. muricata*, dull green in *A. atemoya* and light green rind covered with whitish bloom in different genotypes of *A. squamosa* except for Red Sitaphal, which possessed dark rind covered with violet bloom (Table 4.4).

#### **4.1.3.9 Colour of fruit (pulp)**

Fruit pulp colour varied from creamy white (*A. cherimola*, *A. atemoya* and genotypes of *A. squamosa*), milky white (*A. reticulata* and *A. muricata*) to creamy white with reddish tinge (Red Sitaphal).

#### **4.1.3.10 Fruit shape**

Different fruit shapes like heart shaped, conical oval to irregular (*A. cherimola*), heart shaped, conical ovate to irregular (*A. reticulata*), ovate conical or

heart shaped fruit (*A. muricata*), heart shaped conical to irregular (*A. atemoya*) and round heart shaped, ovate or conical in different genotypes of *A. squamosa* were recorded and presented in Table 4.4.

#### **4.1.4 Seed morphology**

*Annona* fruits contained seeds of different shapes, size and colours. A brief summary of results is presented below (Fig 4.8 and 4.9);

##### **4.1.4.1 Seed length (cm)**

Seed length ranged from 1.11 to 1.83 cm in different genotypes (Table 4.3). Significantly higher seed length was recorded for *A. cherimola* (1.83 cm), which was at par with *A. atemoya* (1.79 cm), whereas it was significantly lower for Red Sitaphal (1.11 cm). Seed length for *A. reticulata*, *A. muricata*, Anand Selection, Sindhan, Balanagar and GJCA-1) was found 1.57, 1.57, 1.38, 1.29, 1.29 and 1.27cm, respectively.

As *Annona* seeds contain good amount of oil and important phytochemicals genotypes with high seed length can be selected and utilized in improving *Annona* crop for bold seeds.

##### **4.1.4.2 Seed width (cm)**

Seed width for different genotypes of *Annona* was measured and presented in Table 4.3. Seed width was ranged from 0.52 to 0.88 cm. Significantly higher seed width was recorded for *A. atemoya* (0.88 cm), which was at par with *A. cherimola* (0.83cm).









and *A. muricata* (0.81 cm) and significantly lower seed width was recorded for Red Sitaphal (0.52 cm). Seed width for *A. reticulata*, GJCA-1, Anand Selection, Sindhan and Balanagar was 0.77, 0.72, 0.67, 0.64 and 0.62cm, respectively.

Above result suggested the existence of higher diversity for seed width among *Annona* genotypes. Crop improvement for bold seeds can be possible by using genotypes with higher seed width.

#### **4.1.4.3 Number of seeds per fruit**

A range of 38 to 49 seeds per plant was recorded in different genotypes of *Annona* species (Table 4.3). Significantly higher number of seeds per fruit was recorded for Balanagar (49), which was at par with *A. muricata* (48) and Sindhan (48), whereas significantly lower number of seeds per fruit was recorded for Red Sitaphal (38). Number of seeds per fruit recorded for *A. atemoya*, GJCA-1, *A. reticulata*, Anand Selection and *A. cherimola* were 45, 45, 44, 43 and 42, respectively.

Above result revealed the presence of considerable amount of variation, hence crop improvement for higher seed numbers can be done by utilizing genotypes with higher no. of seeds.

#### **4.1.4.4 100 seed weight (g)**

Weight of hundred seeds was recorded for different genotypes of *Annona* (Table 4.3). Hundred seed weight was ranged from 12.67 to 41.30 g. Significantly higher weight of 100 seeds was recorded for *A. atemoya* (41.30 g), while Red

Sitaphal showed significantly lower 100 seed weight (12.67 g). Weight of 100 seeds for *A. muricata*, *A. cherimola*, *A. reticulata*, GJCA-1, Sindhan, Anand Selection and Balanagar was found 38.04, 37.59, 27.77, 19.52, 17.73, 16.18 and 14.72 g, respectively.

The above result indicated that there is wide diversity among *Annona* genotypes for this trait.

#### **4.1.4.5 Seed colour**

Seed colour varied from brown to black (*A. cherimola*), brown to dark coffee coloured (*A. reticulata*), brown to dark brown (*A. muricata* and *A. atemoya*), whereas different genotypes of *A. squamosa* possessed shiny brown to black coloured seeds (Table 4.4).

#### **4.1.4.6 Seed shape**

Seed shape varied from oblong to oval (*A. cherimola*, *A. muricata* and *A. atemoya*), whereas oblong shaped seeds were recorded in *A. reticulata*. Different genotypes of *A. squamosa* possessed oblong shaped seeds (Table 4.4).

### **4.1.5 Genetic variability components for different morphological parameters**

#### **4.1.5.1 Leaf length**

Estimates of GCV (43.85%) and PCV (44.41%) were high, indicating the existence of high variability for leaf length. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.





High value of heritability (97.5%) coupled with high genetic advance (89.14%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.2 Leaf width**

High estimates of GCV (61.57%) and PCV (62.12%) indicated existence of high variability for leaf width. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (98.2%) coupled with high genetic advance (98.0%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.3 Length of petiole**

Estimates of GCV (44.74%) and PCV (45.34%) were high, which indicated the existence of high variability for the length of petiole. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (97.4%) coupled with high genetic advance (90.67%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.4 Petal length**

High estimates of GCV (20.61%) and PCV (21.27%) indicated the existence of high variability for petal length. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (93.9%) coupled with high genetic advance (41.23%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.5 Petal width**

Estimates of GCV (29.40%) and PCV (29.69%) were high, which indicated the existence of high variability for the length of petal width. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (98.1%) coupled with high genetic advance (59.77%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.6 Length of pedicel**

High estimates of GCV (31.50%) and PCV (31.97%) indicated the existence of high variability for length of pedicel. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (97.0%) coupled with high genetic advance (63.86%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.7 Fruit length**

Estimates of GCV (14.18%) and PCV (15.48%) were moderate to high, which indicated the existence of variability for the length of fruit length. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (83.9%) coupled with high genetic advance (26.71%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.8 Fruit width**

Moderate estimates of GCV (12.57%) and PCV (13.36%) indicated the existence of moderate variability for fruit width. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (84.8%) coupled with high genetic advance (23.87%) indicated that this trait is under the influence of additive gene action and

hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.9 Fruit weight**

Estimates of GCV (41.33%) and PCV (42.54%) were high, which indicated the existence of variability for the length of fruit weight. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (94.4%) coupled with high genetic advance (82.55%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.10 Fruit volume**

High estimates of GCV (37.86%) and PCV (38.43%) indicated the existence of high variability for fruit volume. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (97.1%) coupled with high genetic advance (76.78%) indicated that this trait is under strong influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.11 Fruit density**

Estimates of GCV (4.49%) and PCV (6.81%) were low, which indicated the existence of very less amount of variability for the length of fruit density. Least difference between GCV and PCV values suggested less influence of the environment for the expression of the character.

Moderate value of heritability (43.5%) coupled with low genetic advance (5.76%) indicated that simple selection based on these traits will be inefficient (Table 4.5).

#### **4.1.5.12 Number of fruits per plant**

High estimates of GCV (25.99%) and PCV (27.38%) indicated the existence of high variability for no. of fruits per plant. Least difference between GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (90.1%) coupled with high genetic advance (50.80%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.13 Seed length**

Estimates of GCV (17.55%) and PCV (18.36%) were moderate to high, which indicated the existence of moderate amount of variability for the seed length.

Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (91.4%) coupled with high genetic advance (34.45%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.14 Seed width**

Moderate estimates of GCV (15.85%) and PCV (16.75%) indicated the existence of moderate amount of variability for no. of seed width. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (89.5%) coupled with high genetic advance (30.55%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.15 Number of seeds per fruit**

Estimates of GCV (12.03%) and PCV (15.56%) were moderate to high, which indicated the existence of moderate amount of variability for seeds per fruit among different genotypes. Least difference between GCV and PCV values suggested less contribution of the environment for the expression of the character.

Moderate value of heritability (59.8%) coupled with moderate genetic advance (19.64%) indicated that this trait is under influence of additive gene action and hence simple selection based on phenotypic performance of these traits will be effective (Table 4.5).

#### **4.1.5.16 Hundred seed weight**

High estimates of GCV (44.87%) and PCV (45.47%) indicated the existence of high amount of variability for no. of hundred seed weight. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (97.4%) coupled with high genetic advance (91.18%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.5.17 Fruit yield (Kg) per plant**

Estimates of GCV (35.17%) and PCV (37.13%) were high, which indicated the existence of high amount of variability for Kg fruit yield per plant. Least difference between GCV and PCV values suggested less contribution of the environment for the expression of the character.

High value of heritability (89.7%) coupled with high genetic advance (68.64%) indicated that this trait is under the influence of additive gene action and

hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.5).

#### **4.1.6 Correlation analysis for morphological parameters**

Prior to any breeding programme for the improvement of crops for any trait, it is imperative to obtain information regarding the inter relationship of different characters among themselves and with yield, since it facilitates quicker assessment of high yielding genotypes in selection programme. The real or true association could be known through genotypic correlation, which eliminates the environmental influence.

The phenotypic and genotypic correlation coefficients were estimated among all 17 characters of 9 *Annona* genotypes to find out the association among these characters. The estimated values of genotypic and phenotypic correlations are presented in the Table 4.6. The results of correlation study revealed comparatively higher degree of genotypic correlation coefficient than their phenotypic counterpart in all the characters, which indicated that there was a strong and inherent association between two characters without influence of any non genetic cause or environmental factor.

##### **4.1.6.1 Leaf length**

Significant positive genotypic and phenotypic correlation of leaf length was observed with leaf width, length of petiole, fruit length, fruit width, fruit weight, fruit volume, seed length, seed width, hundred seed weight and fruit yield per plant,



while it showed negative association with no. of fruits per plant although it was not significant (Table 4.6).

Positive significant correlation suggested that more leaf length cause increase in fruit length, fruit width, fruit volume, fruit weight and fruit yield per plant.

#### **4.1.6.2 Leaf width**

Leaf width showed significant positive genotypic as well as phenotypic correlation with length of petiole, fruit length, fruit width, fruit weight, fruit volume, seed length, seed width, hundred seed weight and fruit yield per plant, while it showed negative and non significant association with no. of fruits per plant.

Positive significant correlation indicated that more leaf width cause increase in fruit length, fruit width, fruit volume, fruit weight and fruit yield per plant.

#### **4.1.6.3 Length of petiole**

Length of petiole possessed significantly positive correlation with fruit length, fruit width, fruit weight, fruit volume, seed length, seed width, hundred seed weight and fruit yield per plant at both genotypic as well as phenotypic level, while it showed negative correlation with no. of fruits per plant although it was non significant (Table 4.6).

Positive significant correlation indicated that length of petiole contributed positively towards increase in fruit length, fruit width, fruit volume, fruit weight and fruit yield per plant.

#### **4.1.6.4 Petal length**

There was significant positive genotypic and phenotypic association of petal length with fruit width, whereas it was significant and positively correlated with fruit weight, fruit volume and fruit yield per plant only at phenotypic level (Table 4.6).

Positive significant correlation indicated that length of petiole contributed positively towards increase in fruit length, fruit width, fruit weight, fruit volume and fruit yield per plant.

#### **4.1.6.5 Petal width**

Significant positive genotypic and phenotypic correlation of petal width was observed only with fruit length, while it showed negative association with fruit yield per plant although it was not significant (Table 4.6).

#### **4.1.6.6 Length of pedicel**

Length of pedicel was found to be significant positively correlated fruit yield per plant at both genotypic as well as phenotypic level, while it showed significant positive phenotypic association fruit width, fruit weight and fruit volume (Table 4.6).

#### **4.1.6.7 Fruit length**

Significant positive genotypic and phenotypic correlation of fruit length was observed with fruit weight, fruit volume, fruit density, seed length, seed width, hundred seed weight and fruit yield per plant, while it showed significant positive

phenotypic association with fruit width. Significant negative association of fruit length was found with no. of fruits per plant at both genotypic and phenotypic levels (Table 4.6).

Positive significant correlation suggested that fruits with more length will also possess more fruit weight, fruit volume, fruit density, seed length, seed width, hundred seed weight and fruit yield per plant, whereas negative significant association indicated decrease in the no. of fruit per plant with increase in the fruit length which is a typical feature of fruit trees.

This correlation clearly suggested that we have to compromise with one character if we want other character more. Simultaneous improvement of both the characters is not possible at a time.

#### **4.1.6.8 Fruit width**

Fruit length showed significant positive genotypic and phenotypic association with fruit weight, fruit volume and fruit yield per plant, while it showed significant positive phenotypic association with seed length, seed width and hundred seed weight (Table 4.6).

Positive significant correlation suggested that fruits with more width will also have more fruit weight, fruit volume and fruit yield per plant.

#### **4.1.6.9 Fruit weight**

Fruit weight possessed significantly positive correlation with fruit volume, seed length, seed width, hundred seed weight and fruit yield per plant at both genotypic as well as phenotypic level, while it showed negative correlation with no. of fruits per plant which was significant at phenotypic level only (Table 4.6).

Positive significant correlation suggested that more the fruit weight more will be fruit volume, seed length, seed width and fruit yield per plant, whereas negative association showed no. of fruits per plant will be less if there is more fruit weight.

#### **4.1.6.10 Fruit volume**

Fruit volume showed significant positive genotypic as well as phenotypic correlation with seed length, seed width and hundred seed weight, while it showed positive and significantly associated with fruit yield per plant only at phenotypic level. Negative association was found with no. of fruits per plant which was significant only at phenotypic level (Table 4.6).

Positive significant correlation indicated that fruits with more volume will also have more seed length, seed width and hundred seed weight, whereas negative association suggested that no. of fruits per plant will reduce if fruit volume is more.

#### **4.1.6.11 Fruit density**

There was significant positive genotypic and phenotypic association of fruit density with seed length, seed width and hundred seed weight, whereas it was

negatively correlated with no. of fruits per plant although it was non significant (Table 4.6).

Positive significant correlation indicated that there will be increased seed length, seed width, hundred seed weight, if there is more fruit density.

#### **4.1.6.12 Number of fruits per plant**

Significant positive genotypic and phenotypic association of no. of fruits per plant was found with fruit yield per plant, whereas it was negatively correlated with seed length, seed width, no. of seeds per fruit and hundred seed weight, although it was non significant (Table 4.6).

Positive significant correlation indicated that if there is more no. of fruits per plant, there will be more fruit yield per plant.

#### **4.1.6.13 Seed length**

Seed length was observed significant positive genotypically and phenotypically correlated with seed width and hundred seed weight, while it showed significant positive phenotypic association with fruit yield per plant (Table 4.6).

Positive significant correlation suggested that seeds with more length will have more seed width and hundred seed weight. It was indirectly related with fruit yield per plant.

#### **4.1.6.14 Seed width**

Seed width was observed significant positive genotypically and phenotypically correlated hundred seed weight, while it showed significant positive phenotypic association with fruit yield per plant (Table 4.6).

Positive significant correlation suggested that seeds with more width will have more seed weight and hundred seed weight. It was indirectly related with fruit yield per plant.

#### **4.1.6.15 Number of seeds per fruit**

No. of seeds per fruit did not show any significant correlation, however it was correlated with hundred seed weight and fruit yield per plant (Table 4.6).

#### **4.1.6.16 Hundred seed weight**

Hundred seed weight was significant positively correlated with fruit yield per plant but only at phenotypic level. It was non significant and indirectly related with fruit yield per plant (Table 4.6).

### **4.2 BIOCHEMICAL CHARACTERIZATION**

#### **4.2.1 Qualitative parameters/proximate composition**

Various qualitative parameters have been studied for all nine genotypes of *Annona* with three replications. The mean data obtained for all the parameters is presented in Table 4.8-4.9. The results obtained in the present study are given as follows;

#### **4.2.1.1 Moisture content (%)**

Moisture deficiency is essential for good quality and quantity of the yield. The severe deficiency may lead to the death of plant.

In this experiment, moisture content was measured in the fruits of different *Annona* genotypes. The moisture content was ranged from 72.55 to 81.23 percent (Table 4.7a and Fig 4.11). Significantly higher moisture content was recorded for *A. muricata* (81.23%), which was statistically at par with *A. cherimola* (79.26%) and *A. atemoya* (76.63%). Significantly lower moisture content (72.55%) was recorded for GJCA-1.

Similar result of 81% moisture content was claimed by Onimawo (2002) in the fruit pulp of *A. muricata*. Pareek *et al.* (2011) also confirmed  $77.3 \pm 3$ ,  $75.8 \pm 2.8$ ,  $81 \pm 2.5$  and  $72.6 \pm 2.4$  g moisture content in the edible pulp of cherimola, custard apple, sour sop and sugar apple, respectively.

The experimental result revealed that *A. muricata* followed by *A. cherimola* and *A. atemoya* contained relatively more moisture content, thus can be more beneficial as moisture content makes the fresh fruit juicier and more palatable to the consumer.

#### **4.2.1.2 Ash content (%)**

High amount of ash content indicates the presence of high mineral elements. Ash content was measured for the fruits of different *Annona* genotypes (Table 4.7a and Fig 4.12). Among different *Annona* cultivars maximum ash content was





recorded in case of *A. muricata* (1.69%), which was statistically at par with *A. cherimola* (1.56%) and *A. atemoya* (1.62%). The minimum ash content was recorded for Anand Selection, Balanagar and GJCA-1 (1.32%), which was at par with Red Sitaphal (1.41%) and Sindhan (1.43%).

This result matched with Pareek *et al.* (2011) who claimed edible pulp of cherimola, custard apple, sour sop and sugar apple to contain  $0.7 \pm 0.1$ ,  $0.95 \pm 0.15$ ,  $0.61 \pm 0.2$  and  $0.7 \pm 0.1$  g ash content, respectively.

According to the above result it can be suggested that *A. muricata*, *A. atemoya* and *A. cherimola* contained relatively higher ash content, which indicating presence of relatively higher amount of mineral elements in the fruits.

#### **4.2.1.3 Total carbohydrate content (%)**

Carbohydrates serve organisms as energy sources and as essential structural component of nucleic acid which stores genetic information.

Total carbohydrate content was measured for the fruits of different genotypes of *Annona*. Total carbohydrate content was ranged from 16.48 to 24.05 percent (Table 4.7b and Fig 4.13). Genotype GJCA-1 contained significantly higher amount of total carbohydrates (24.05%) followed by Balanagar (23.95%), which was at par with *A. reticulata* (22.71%), Red Sitaphal (22.51%), Anand Selection (22.66%) and Sindhan (23.04%). Significantly lower amount of total carbohydrates was recorded for *A. muricata* (16.48%). Carbohydrates for *A. cherimola*, *A. atemoya* was found 17.54 and 20.7%, respectively.



The above experimental result revealed that genotypes of *A. squamosa* contained more amount of carbohydrates, which means they are the potential source of energy in the diet.

Pareek *et al.* (2011) also obtained  $18.42 \pm 4$ , 18.7,  $17.25 \pm 0.1$  and  $19.6 \pm 1$  g of carbohydrate content in the pulp of cherimola, custard apple, sour sop and sugar apple, respectively.

#### **4.2.1.4 Total soluble sugars (%)**

Sugars play important role in osmotic adjustment and in providing protection against various types of stresses.

TSS content was measured in the fruit pulps of different genotypes of *Annona*. TSS found in the range of 8.92 to 17.44% (Table 4.7b and Fig 4.14). Maximum amount of total soluble sugars was recorded in case of Balanagar (17.44%), which was at par with Anand Selection (16.29%), Sindhan (16.69%) and GJCA-1 (16.63%), whereas minimum amount of total soluble sugars was recorded for *A. muricata* (8.92%). Total soluble sugar was 14.41, 22.71, 14.62 and 22.51% for *A. cherimola*, *A. reticulata*, *A. atemoya* and Red Sitaphal, respectively.

Above result suggested that genotypes of *A. squamosa* possess fruits with high content of total soluble sugars.



#### 4.2.1.5 Reducing sugar content (%)

Sugars are the primary products of photosynthesis and perform multiple roles in plants as energy and carbon transport molecules, hormone like signaling factors, from which plants make proteins, polysaccharides, oils and woody materials. It is also responsible for resistance mechanism against biotic stresses.

In this experiment, reducing sugars was measured in the fruits of different *Annona* genotypes. Reducing sugar content was found in the range of 3.27 to 8.39 percent (Table 4.7b and Fig 4.15). Reducing sugar was maximum for Balanagar (8.39%), whereas minimum amount of reducing sugar was recorded for *A. muricata* (3.27%). Genotypes like *A. cherimola*, *A. reticulata*, *A. atemoya*, Red Sitaphal, Anand Selection, Sindhan and GJCA-1 possessed 4.33, 4.80, 6.67, 7.49, 7.61, 7.81 and 7.75% reducing sugar, respectively.

Benkeblia *et al.* (2014) also reported similar result of 11.98 to 17.88 mg reducing sugar per gram of fresh weight of custard apple (*A. reticulata*) fruit.

It can be concluded from the above result that genotypes of *A. squamosa* contained significantly higher amount of reducing sugar so can be a source of resistance against biotic stresses.

#### 4.2.1.6 Protein content (%)

Proteins are the building block of the body and are important components of various enzymes, nucleic acid etc. Good amount of proteins are required in our daily diet.



Protein content in different genotypes of *Annona* was in the range of 1.15 to 2.14 percent (Table 4.7a and Fig 4.16). Significantly higher protein content was recorded for Balanagar (2.14%), which was found at par with *A. reticulata* (1.85%), *A. atemoya* (1.84%), Anand Selection (1.81%), Sindhan (2.13%) and GJCA-1 (1.98%), whereas it was significantly lower for *A. muricata* (1.15%). Remaining genotypes like *A. cherimola* and Red Sitaphal were found to have 1.69% and 1.78% protein content, respectively.

The similar result of protein content was obtained by Onimawo (2002), who confirmed that soursop (*A. muricata*) seed contained 2.4% crude protein and the pulp contained 1.23 % of proteins per 100 g of fresh weight. Pareek *et al.* (2011) also reported that  $1.6 \pm 0.6$ ,  $1.85 \pm 0.05$ ,  $1 \pm 0.55$  and  $1.68 \pm 0.8$  g of protein content per 100 g of fresh fruit of cherimola, custard apple, sour sop and sugar apple, respectively.

The above results suggested that the fruits of *A. squamosa* cultivars like Sindhan, Balanagar and GJCA-1 can be good source of proteins in consumer's diet.

#### **4.2.1.7 Fibre content (%)**

The dietary fibre plays an important role in decreasing the risk of many disorders such as constipation, diabetes, cardiovascular diseases and obesity.

Fibre content was measured in the fruits of different genotypes of *Annona* species. Fibre content in fruits was found in the range of 2.18 to 3.82 percent (Table

4.7b and Fig 4.17). *A. atemoya* possessed significantly higher amount of fibre content (3.82%), followed by Sindhan (3.38%), GJCA-1 (3.32%), Red Sitaphal (3.28%), Balanagar (3.24%) and Anand Selection (3.18%). Significantly lower fibre content was recorded for *A. reticulata* (2.18%), which was at par with *A. cherimola* (2.22%) and *A. muricata* (2.34%).

Similar result was claimed by Pareek *et al.* (2011), who obtained  $1.64 \pm 0.5$ ,  $2.55 \pm 0.35$ ,  $0.86 \pm 0.1$  and  $1.4 \pm 0.6$  g of edible fibres in the fruit pulps of cherimola, custard apple, sour sop and sugar apple, respectively. Othman (2014) also reported 3.11 - 6.09 mg crude fibres per 100 g fresh weight of soursop fruit.

Results of present study revealed that *Annona* species especially *A. atemoya* and *A. squamosa* contained relatively more fibres and thus, they can be more helpful for maintaining the health of the gastro-intestinal tract, weight regulation and thus decreasing the risk of many disorders.

#### **4.2.1.8 Phenol content (%)**

Phenolic compounds are plant secondary metabolites which constitute one of the most common and widespread group of compounds in plants. Plants need phenolic compounds for pigmentation, growth, reproduction, resistance to diseases and many other functions.

Fruits of different genotypes of *Annona* species were studied for phenol content. Phenol content was recorded in the range of 0.26 to 0.50 percent (Table



4.7a and Fig 4.18). Significantly higher phenol content was recorded for *A. muricata* (0.50%) followed by *A. reticulata* (0.41%), *A. atemoya* (0.40%), *A. cherimola* (0.35%) and Red Sitaphal (0.34%), whereas it significantly lower for Sindhan (0.26%) which was at par with Anand Selection (0.31%), Balanagar (0.29%) and GJCA-1 (0.30%).

Study of Benkeblia *et al.* (2014) also revealed 9.83 to 18.71 mg phenols per gram of fruit pulp of custard apple (*A. reticulata*).

Above result indicated that *A. muricata* which possessed significantly higher phenol content can be a potential source of disease resistance and also for therapeutic purpose in pharmaceuticals.

#### **4.2.1.9 Acidity (%)**

Titrateable acidity content was estimated from the fruits of different *Annona* genotypes. A range of 0.17 to 0.65 percent of titrateable acidity was recorded in different genotypes (Table 4.7a and Fig 4.19). Titrateable acidity was found maximum in *A. muricata* (0.65%) followed by *A. reticulata* (0.38%), *A. atemoya* (0.37%), *A. cherimola* (0.36%), Red Sitaphal (0.29%), Anand Selection (0.24%) and Sindhan (0.23%), whereas titrateable acidity was minimum for Balanagar (0.17%) which was at par with GJCA-1 (0.19%).

In comparison to above result, Onimawo *et al.* (2002) obtained slightly higher amount of titrateable acidity 0.79 and 3.43% in seeds and pulp of soursop (*A.*

*muricata*), respectively. Study of Othman *et al.* (2014) also revealed titratable acidity ranging from 0.10-1.25% in freshly matured fruit.

Because acidity in fruits plays an important role in taste, color, and microbial stability of the fruit juice and determines maturity it can be concluded that *Annona* fruits can have better acceptability for the consumers.

#### **4.2.1.10 Seed oil content (%)**

Estimation of oil content from seeds of different genotypes of *Annona* species was done by using Soxhlet apparatus. Seed oil content was found in the range of 23.13 to 32.50 percent (Table 4.7a and Fig 4.20). Significantly higher seed oil content was obtained for *A. muricata* (32.50%) followed by *A. cherimola* (29.33%), *A. reticulata* (29.05%), *A. atemoya* (28.41%), Sindhan (26.56%), Balanagar (26.52%) and GJCA-1 (26.35%). Seed oil content was significantly lower for Red Sitaphal (23.13%) which was at par with Anand Selection (24.41%).

The above result was compared with Mariod *et al.* (2010) who claimed 26.8% oil content in the seeds of *A. squamosa* and Rana (2015), who recorded 22.2% of crude fatty oil in the seed kernels of *A. squamosa* on a dry weight basis.

These results indicated that *Annona* seeds could be very useful as a source of oil for both domestic and industrial uses because of the relatively high oil content. Instead of discarding them as waste they can be better utilized in oil industries and can also be used as cooking oil if it is deodorized.



#### 4.2.1.11 Ascorbic acid content (%)

Ascorbic acid content was measured in the fruits of different genotypes of *Annona* species. Ascorbic acid content was found in the range of 19.60 to 39.24 percent (Table 4.7a and Fig 4.21). Maximum content of ascorbic acid was recorded for *A. muricata* (39.24%) followed by Balanagar (33.39%), *A. atemoya* (32.74%), Anand Selection (32.29%), Sindhan (32.06%), GJCA-1 (31.57%), Red Sitaphal (28.41%) and *A. reticulata* (23.36%), whereas minimum ascorbic acid content was recorded for *A. cherimola* (19.60%).

Pareek *et al.* (2011) also claimed  $11.5 \pm 5.5$ , 30.0,  $29.4 \pm 3$  and  $37.38 \pm 4.62$ mg of ascorbic acid per 100g of pulp of cherimola, custard apple, sour sop and sugar apple, respectively. A range of 34.0 – 19.7mg ascorbic acid per 100g fresh weight of soursop fruits was recorded by Othman *et al.* (2014), while Boake *et al.* (2014) obtained 20.33 and 63.67 mg ascorbic acid per 100 g of sweetsop and soursop fruits, respectively.

The high values of ascorbic acid in *Annona* signify the potential use of the fruit as a good source of ascorbic acid. The recommended daily intake (RDI) of ascorbic acid is about 30 mg/day for adults and 17 mg/day for children. Therefore, these fruits could be considered as good sources of ascorbic acid for purposes of human nutrition.



#### **4.2.2 Antioxidant activities**

The antioxidant activity is an essential biological property of great interest because it reduces the toxic effects of oxidants and thus prevents the cellular damage caused by free radicals. In present investigation, the antioxidant activity was studied in pulps and seeds of different genotypes of *Annona* species. The results obtained are briefly described below;

##### **4.2.2.1 Screening of antioxidant activity in pulp**

Antioxidant activity of 0.19 to 0.31 percent was recorded in the pulps of different *Annona* genotypes (Table 4.8 and Fig 4.22). Significantly higher antioxidant activity was recorded for *A. muricata* (0.31%), which was statistically at par with *A. atemoya*, while significantly lower antioxidant activity was recorded for Red Sitaphal (0.19%).

##### **4.2.2.2 Screening of antioxidant activity in seed**

Antioxidant activity was screened in the seeds of different genotypes and recorded in the range of 0.29 to 0.48 percent (Table 4.8 and Fig 4.23). Significantly higher antioxidant activity was recorded for *A. muricata* (0.48%), whereas *A. cherimola* showed significantly lower antioxidant activity (0.29%) followed by *A. atemoya* (0.43%) and Anand selection (0.41%), respectively.

Mariod *et al.* (2010) and Vijayaraghavan *et al.* (2013) reported 7.81 to 125.0 µg/ml antioxidant activities using DPPH assay in *A. Squamosa* extracts which was slightly higher than the results obtained in the present study.

**Table 4.7 Screening of antioxidant activity (%) in seeds and pulps of *Annona* genotypes**

| S.No. | Genotypes            | Antioxidant activity in Seed (%) | Antioxidant activity in pulp (%) |
|-------|----------------------|----------------------------------|----------------------------------|
| 1.    | <i>A. cherimoya</i>  | 0.29                             | 0.23                             |
| 2.    | <i>A. reticulata</i> | 0.33                             | 0.24                             |
| 3.    | <i>A. muricata</i>   | <b>0.48</b>                      | <b>0.31</b>                      |
| 4.    | <i>A. atemoya</i>    | 0.43                             | 0.29                             |
| 5.    | Red Sitaphal         | 0.38                             | 0.19                             |
| 6.    | Anand selection      | 0.41                             | 0.21                             |
| 7.    | Sindhan              | 0.38                             | 0.21                             |
| 8.    | Balanagar            | 0.38                             | 0.22                             |
| 9.    | GJCA-1               | 0.39                             | 0.21                             |
|       | S.Em.±               | 0.01                             | 0.01                             |
|       | C.D. @ 5%            | 0.04                             | 0.04                             |
|       | C.V. %               | 6.22                             | 9.31                             |

*Annona* species seeds of different genotypes of *Annona* contained relatively more amount of antioxidant activity as compared to fruit pulp indicating that seeds are less prone to chemical oxidation due to free radicals as compared to fruit pulp.

From the study it can be revealed that species of *Annona* viz., *A. cherimola*, *A. reticulata*, *A. muricata*, *A. atemoya* and different cultivars of *A. squamosa* can act as important source of compounds with a high potential to protect health. *Annona* species especially *A. muricata* possessed relatively higher amount of antioxidants but unfortunately the fruits of this species are not sweet.



Fruits of species like *A. cherimola*, *A. atemoya* and *A. squamosa* were sweet enough and also possessed higher quantity of antioxidants. Thus, it can be suggested that the fruits of these species can be good source of natural antioxidants.

#### **4.2.3 Determination of phytochemicals**

*Annona* are rich in phenolic compounds and are very useful for their therapeutic potentials.

The present study was carried out for qualitative analysis of secondary metabolites in the fruits and seeds of different *Annona* genotypes. The qualitative analysis of powdered extracts of pulp and seeds was carried out with reference to phytochemicals like steroids, terpenoids, tannins, saponins, alkaloids, flavenoids, phenols and glycosides *etc.* (Table 4.9 and 4.10).

The results obtained by preliminary phytochemical screening of different species of *Annona* are summarized below;

##### **4.2.3.1Determination of steroids:**

**(i) Pulp:-**Steroid was found maximum in the pulp of *A. muricata* and *A. reticulata*. Remaining all genotypes showed the presence of steroids but in lesser quantity.

**(ii) Seed:-***A. muricata* and *A. reticulata* seeds also showed more quantity of steroids as compared to other genotypes of *Annona* species. (Fig 4.24)



#### 4.2.3.2 Determination of terpenoids:

(i) **Pulp:-**Terpenoids were recorded maximum in the pulps of *A. muricata* followed by different genotypes of *A. squamosa* and *A. cherimola*. It was found absent in *A. reticulata* (Fig 4.25).

(ii) **Seed:-**Seeds of *A. muricata*, *A. squamosa* and *A. cherimola* showed more amount of terpenoids as compared to pulps. Terpenoids were found absent in seeds of *A. reticulata*.

#### 4.2.3.3 Determination of tannins:

(i) **Pulp:-** The pulps of *A. cherimola* and *A. muricata* possessed more quantity of tannins as compared to *A. atemoya* and genotypes of *A. squamosa*. Pulp of *A. reticulata* showed the absence of tannins (Fig 4.27).

(ii) **Seed:-***A. cherimola* and *A. muricata* seeds possessed more quantity of tannins as compared to seeds of *A. atemoya* and genotypes of *A. squamosa*. Tannins were found absent in seeds of *A. reticulata*.

#### 4.2.3.4 Determination of saponin:

(i)**Pulp:-**Saponin was found absent in the pulps of all the genotypes.

(ii) **Seed:-**Saponin was found absent in the seeds of all the genotypes.

#### 4.2.3.5 Determination of alkaloids:

(i) **Pulp:-**Alkaloids were recorded maximum in the pulps of *A. muricata* followed by genotypes of *A. squamosa* and *A. cherimola*.



among *A. squamosa*, GJCA-1 genotype showed maximum alkaloids, whereas it was found absent in *A. reticulata*.

**(ii) Seed:-**Seeds of *A. muricata* showed maximum amount of alkaloids followed by genotypes of *A. squamosa* and *A. cherimola*. Among genotypes of *A. squamosa*, GJCA-1 showed maximum alkaloids. *A. reticulata* seeds were found absent in alkaloids (Fig 4.26).

#### 4.2.3.6 Determination of flavenoids:

**(i) Pulp:-**Flavenoid content was found in a very less quantity. *A. cherimola* showed relatively more amount of flavenoids followed by *A. atemoya* as compared to *A. muricata* and *A. squamosa*. Whereas it was found absent in the pulp of *A. reticulata*.

**(ii) Seed:-**Seeds showed relatively more flavenoid content as compared to pulps of different genotypes. The concentration pattern flavenoids was almost similar to pulp. Flavenoids were also found absent in seeds of *A. reticulata*.

#### 4.2.3.7 Determination of phenols:

**(i) Pulp-** More phenol content was recorded in the pulps of *A. muricata* followed by *A. reticulata* and *A. cherimola* and all the genotypes of *A. squamosa* showed relatively lesser quantity of phenols as compared to above genotypes.

**(ii) Seed-** Phenol content was recorded maximum in the seeds of *A. muricata* followed by *A. reticulata* and *A. cherimola*. In comparison, *A. squamosa* seeds showed relatively lesser quantity of phenol.

#### **4.2.3.8 Determination of glycosides:**

**(i) Pulp-** Glycoside was found absent in the pulps of all the genotypes.

**(ii) Seed-** Glycoside was found absent in the seeds of all the genotypes.

The above result was compared with the studies of Savithramma *et al.* (2011), Soni *et al.* (2011), Florence *et al.* (2013) and Biba *et al.* (2013), who claimed the presence of steroids, terpenoids, tannins, alkaloids, flavenoids and phenols in *Annona* species.

Thus, the results obtained in the present study indicated that species of *Annona viz.*, *A. cherimola*, *A. reticulata*, *A. muricata*, *A. atemoya* and different cultivars of *A. squamosa* have the potential to act as a source of useful drugs because of presence of various phytochemical components such as carbohydrates, proteins, lipids, phenols, alkaloids, steroids, flavenoids, terpenoids and tannins.

*Annona* species especially *A. muricata* possessed relatively higher amount of phytochemicals may be due to its wild nature. *A. cherimola* and *A. atemoya* also possessed higher quantity of phytochemicals. Thus, it can be suggested that species *A. muricata* can be useful for the improvement of other *Annona* species.

#### **4.2.4 Genetic variability components for different biochemical parameters**

##### **4.2.4.1 Moisture content**

Estimates of GCV (3.09%) and PCV (5.14%) were low, indicating the existence of low variability for moisture content. Higher difference between GCV



and PCV values suggested more contribution of the environment for the expression of the character.

Low value of heritability (36.2%) coupled with low genetic advance (3.82%) indicated that simple selection based on these traits will be inefficient, thus very difficult to improve these traits through common breeding methods (Table 4.11).

#### **4.2.4.2 Ash content**

Low estimates of GCV (8.81%) and PCV (10.48%) indicated existence of low variability for ash content. Least difference between GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (70.7%) coupled with moderate genetic advance (15.06%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be effective (Table 4.11).

#### **4.2.4.3 Total carbohydrates**

Estimates of GCV (12.30%) and PCV (13.60%) were high, which indicated the existence of high variability for total carbohydrates. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (81.8%) coupled with high genetic advance (22.90%) indicated that this trait is under the influence of additive gene action and

hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.11).

#### **4.2.4.4 Total soluble sugars**

Moderate estimates of GCV (16.57%) and PCV (17.32%) indicated the existence of moderate variability for TSS content. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (91.6%) coupled with high genetic advance (32.69%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.11).

#### **4.2.4.5 Reducing sugars**

High estimates of GCV (28.37%) and high estimates of PCV (28.70%) suggested the existence of high variability for reducing sugars. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (97.8%) coupled with high genetic advance (57.82%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.11).

#### **4.2.4.6 Protein content**

Moderate estimates of GCV (15.03%) and PCV (18.46%) indicated the existence of moderate variability for protein content. Least difference between GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (66.3%) coupled with high genetic advance (25.27%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.11).

#### **4.2.4.7 Fibre content**

Estimates of GCV (19.30%) and PCV (20.05%) were moderate, which indicated the existence of moderate variability for fibre content. Close estimate of GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (87.3%) coupled with high genetic advance (37.12%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.11).

#### **4.2.4.8 Phenol content**

High estimate values of GCV (44.96%) and PCV (45.97%) indicated the existence of moderate variability for phenol content. Close estimate of GCV and

PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (95.6%) coupled with high genetic advance (82.85%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.11).

#### **4.2.4.9 Titratable acidity**

Estimates of GCV (20.81%) and PCV (22.62%) were moderate, which indicated the existence of moderate variability for titratable acidity. Least difference between GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (84.7%) coupled with high genetic advance (43.75%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.11).

#### **4.2.4.10 Seed oil content**

Low to moderate estimates of GCV (9.56%) and PCV (11.59%) indicated the existence of moderate variability for seed oil content. Least difference between GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (68.0%) coupled with moderate genetic advance (16.22%) indicated that this trait is under the influence of non additive gene action and hence simple selection for these traits will be ineffective. Heterosis breeding and population improvement methods will be can be helpful for the improvement of the trait (Table 4.11).

#### **4.2.4.11 Ascorbic acid**

Estimates of GCV (18.79%) and PCV (19.94%) were moderate, which indicated the existence of moderate amount of variability for ascorbic acid content. Close estimate of GCV and PCV values suggested less influence of the environment for the expression of the character.

High value of heritability (88.8%) coupled with high genetic advance (36.49%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.11).

#### **4.2.4.12 Seed antioxidant activity**

Moderate estimates of GCV (14.16%) and PCV (15.47%) indicated the existence of low variability for seed antioxidant activity. Least difference between GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (83.9%) coupled with high genetic advance (25.64%) indicated that this trait is under the influence of additive gene action and

hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.11).

#### **4.2.4.13 Pulp antioxidant activity**

Estimates of GCV (16.45%) and PCV (18.91%) were moderate, which indicated the existence of moderate amount of variability for pulp antioxidant activity. Least difference between GCV and PCV values suggested little contribution of the environment for the expression of the character.

High value of heritability (78.5%) coupled with high genetic advance (30.43%) indicated that this trait is under the influence of additive gene action and hence simple selection based on phenotypic performance of these traits would be more effective (Table 4.11).

#### **4.2.5 Correlation analysis for biochemical parameters**

Prior to any crop improvement programme for any trait, it is always suggested to obtain information regarding the inter relationship of different characters among themselves and with yield, since it facilitates quicker assessment of high yielding genotypes in selection programme. The real or true association could be known through genotypic correlation, which eliminates the environmental influence.

The phenotypic and genotypic correlation coefficients were estimated among all 13 biochemical characters of 9 *Annona* genotypes, to find out the association among these characters. The estimated values of genotypic and phenotypic



correlations are presented in the table 4.6. The results of correlation study revealed comparatively higher degree of genotypic correlation coefficient than their phenotypic counterpart in all the characters, which indicated that there was a strong and inherent association between two characters without influence of any non genetic cause or environmental factor.

#### **4.2.5.1 Moisture content**

Significant positive genotypic and phenotypic correlation of moisture content was observed with ash content, phenol content, titratable acidity, seed oil content and pulp antioxidant activity, while it was significant negatively correlated with total carbohydrates, total soluble sugars, reducing sugars and protein content both at genotypic and phenotypic level. It also showed positive association with fruit yield per plant although it was not significant (Table 4.12).

Positive significant correlation suggested that higher moisture content causes increase in ash content, phenol content, titratable acidity, seed oil content and pulp antioxidant activity and negative association indicated decrease in total carbohydrates, total soluble sugars, reducing sugars and protein content etc. on increasing moisture content.

#### **4.2.5.2 Ash content**

There was a significant positive genotypic and phenotypic correlation of ash content with phenol content, titratable acidity, seed oil content and pulp antioxidant activity, while it was significant negatively correlated with total carbohydrates, total

soluble sugars, reducing sugars and protein content both at genotypic and phenotypic level. It also showed positive association with fruit yield per plant although it was not significant (Table 4.12).

Positive significant correlation suggested that the genotypes which possessed more ash content will also possess higher phenol content, titratable acidity, seed oil content and pulp antioxidant activity and negative association indicated decrease in total carbohydrates, total soluble sugars, reducing sugars and protein content *etc.* on increasing ash content.

#### **4.2.5.3 Total carbohydrates**

Total carbohydrate content was significant and positively associated with total soluble sugars, reducing sugars and protein content both at genotypic and phenotypic levels, while there was significant negative genotypic and phenotypic correlation with phenol content, titratable acidity, seed oil content and pulp antioxidant activity. It also showed positive association with fibre content and fruit yield per plant although it was not significant (Table 4.12).

Positive significant correlation suggested that the genotypes with more total carbohydrates content will also possess higher total soluble sugars, reducing sugars and protein content *etc.* and negative association indicated decrease in phenol content, titratable acidity, seed oil content and pulp antioxidant activity on increasing total carbohydrates.

#### **4.2.5.4 Total soluble sugars**

Significant and positive genotypic and phenotypic correlation for total soluble sugars was found with reducing sugars and protein content, while there was significant negative correlation with phenol content, titratable acidity, seed oil content and pulp antioxidant activity at genotypic and phenotypic levels. It also showed significant positive association with fibre content at phenotypic level and non significant positive association with fruit yield per plant (Table 4.12).

Positive significant correlation suggested that higher TSS content will contribute towards increase in reducing sugars and protein content and negative association indicated decrease in phenol content, titratable acidity, seed oil content and pulp antioxidant activity on increasing TSS.

#### **4.2.5.5 Reducing sugars**

Reducing sugar content was found significant positive genotypic and phenotypically correlated with protein content and fibre content, while it showed negative association with phenol content, titratable acidity, seed oil content and pulp antioxidant activity at genotypic and phenotypic levels (Table 4.12).

#### **4.2.5.6 Protein content**

Protein content was significant and positively associated with fibre content at phenotypic level, while it showed negative association with phenol content, titratable acidity, seed oil content and pulp antioxidant activity at genotypic and phenotypic levels (Table 4.12).

There was a positive correlation of protein content with fruit yield per plant although it was non significant.

#### **4.2.5.7 Fibre content**

Phenotypic correlation of fibre content was significant and negative with phenol content, titratable acidity and seed oil content (Table 4.12).

#### **4.2.5.8 Phenol content**

Significant positive correlation for phenol content was found with titratable acidity, seed oil content and pulp antioxidant activity at genotypic and phenotypic levels (Table 4.12).

Positive significant correlation suggested that genotypes with higher phenol content will also possess higher titratable acidity, seed oil content and pulp antioxidant activity (Table 4.12).

#### **4.2.5.9 Titratable acidity**

Titratable acidity was Significant and positively associated with seed oil content and pulp antioxidant activity at genotypic and phenotypic levels, it was also significant and positively correlated with seed antioxidant activity at phenotypic level only (Table 4.12).

#### **4.2.5.10 Seed oil content**

Significant and positive association of seed oil content was recorded with seed antioxidant activity and pulp antioxidant activity at genotypic and phenotypic

levels, whereas at phenotypic level, it was significant and positively correlated with seed antioxidant activity only (Table 4.12).

#### **4.2.5.11 Ascorbic acid content**

Ascorbic acid content was significant and positively associated with seed antioxidant activity and pulp antioxidant activity at genotypic and phenotypic levels, indicating that genotypes with higher ascorbic acid content will also possess higher seed and pulp antioxidant activity (Table 4.12).

#### **4.2.5.12 Seed antioxidant activity**

Significant and positively correlation was observed for seed antioxidant activity with pulp antioxidant activity at phenotypic level only, suggesting that genotypes with higher seed antioxidant activity will also possess relatively higher pulp antioxidant activity (Table 4.12).

#### **4.2.5.13 Pulp antioxidant activity**

Pulp antioxidant activity was positively correlated with fruit yield per plant but correlation was non significant (Table 4.12).

Most of the above biochemical traits were positive and negatively correlated with fruit yield per plant but none of them possessed significant correction.

### **4.3 MOLECULAR CHARACTERIZATION**

In the last decade, the use of DNA markers for the study of crop genetic diversity has become routine and has revolutionized biology. Increasingly, more

precisely, quickly and cheaper techniques are being developed to assess genetic variation.

The results obtained in the present investigation are presented and discussed in this sub-section under the following sub-headings.

#### **4.3.1 Qualitative and quantitative analysis of genomic DNA**

In present investigation, the average concentration of DNA extracted from *Annona* leaves was 1430.37 ng/μl, quantified on spectrophotometer (Table 4.18). The genotype Vidyanagar local showed the highest DNA concentration of 2829 ng/μl followed by Red Sitaphal showed (2692.2 ng/μl). Lowest concentration of DNA (615.8 ng/μl) was recorded for genotype ACC-5. This difference seems to have occurred only because of handling error during experimentation. There was no correlation observed between yield of DNA and the genotype.

The absorbance ratio of DNA at  $A_{260}/A_{280}$  ranged from 1.78 to 1.91 (Table 4.13). As stated by Sachdev *et al.* (2003) the pure DNA has an  $A_{260}/A_{280}$  ratio of 1.8–2.0. The results in the present investigation were in agreement with the same. Strong absorbance at 280 nm, resulting in a low  $A_{260}/A_{280}$  ratio, indicates the presence of contaminants such as proteins. None of the DNA samples analyzed in the present investigation had poor quality. Quality of DNA was also confirmed on agarose gel which showed unsheared bands. DNA thus extracted was utilized for molecular markers study through RAPD and SSR. The results presented in the next





section are according to the amplification patterns of molecular markers and are described below;

#### **4.3.2 Random Amplification of Polymorphic DNA (RAPD) marker analysis**

The RAPD analysis was carried out in order to analyze the genetic diversity among different *Annona* genotypes as well as to characterize the *Annona* genotypes. As mentioned in previous section, various biostatistical parameters were calculated which are efficient enough for diversity analysis. They included Percent polymorphism, PIC value, PCA (principle component analysis), Matrix Correlation study (Mantel's test) and Similarity index.

RAPD primers of OP series (OPA, OPB, OPD and OPF) were used for preliminary screening and only eleven primers found useful and gave amplified polymorphic pattern (Table 4.15).

##### **4.3.2.1 Individual RAPD primer profile analysis**

Eleven RAPD primers were utilized for characterization of 20 *Annona* genotypes. The results are discussed in the Table 4.15.

##### **4.3.2.1.1 OPB-6**

The banding pattern generated through OPB-6 (Plate 4.1) produced a total of 13 loci, of which 12 loci were polymorphic with 92.30 percent polymorphism. Molecular size of the amplicons ranged from 423 to 1406 bp and the PIC value was 0.89 (Table 4.15).

#### **4.3.2.1.2 OPB-7**

The RAPD marker OPB-7 (Plate 4.2) produced a total of 19 loci, of which 18 loci were polymorphic with 94.73 percent polymorphism. Molecular size of the amplicons ranged from 146 to 1322 bp and the PIC value was 0.92 (Table 4.15).

#### **4.3.2.1.3 OPB-8**

The banding pattern generated through OPB-8 (Plate 4.3) produced a total of 16 loci, of which 14 loci were polymorphic with 87.50 percent polymorphism. Molecular size of the amplicons ranged from 209 to 1328 bp and the PIC value was 0.89 (Table 4.15).

#### **4.3.2.1.4 OPB-10**

OPB-10 (Plate 4.4) RAPD marker produced a total of 15 loci, of which 10 loci were polymorphic with 66.67 percent polymorphism. Molecular size of the amplicons ranged from 294 to 1166 bp and the PIC value 0.92 (Table 4.15).

#### **4.3.2.1.5 OPB-12**

The banding pattern generated through OPB-12 (Plate 4.5) produced a total of 16 loci, of which 15 loci were polymorphic with 93.75 percent polymorphism. Molecular size of the amplicons ranged from 246 to 1391 bp and the PIC value was 0.89 (Table 4.15).

**4.3.2.1.6 OPD-2**

RAPD profile obtained with primer OPD-2 (Plate 4.6) produced ten loci out of which eight loci were found polymorphic with a polymorphism of 80.0 percent. Molecular size of the amplicons ranged from 311 to 1325 bp and the PIC value was 0.86 (Table 4.15).

**4.3.2.1.7 OPD-16**

RAPD marker OPD-16 (Plate 4.7) produced a total of 14 loci, of which 5 loci were polymorphic with 35.71 percent polymorphism. Molecular size of the amplicons ranged from 180 to 1305 bp and the PIC value was 0.92 (Table 4.15).

**4.3.2.1.8 OPD-20**

The banding pattern generated through OPD-20 (Plate 4.8) produced a total of 11 loci, of which 6 loci were polymorphic with 54.54 percent polymorphism. Molecular size of the amplicons ranged from 302 to 1268 bp and the PIC value was 0.89 (Table 4.15).

**4.3.2.1.9 OPF-4**

The banding pattern generated through OPF-4 (Plate 4.9) produced a total of 12 loci, of which 10 loci were polymorphic with 83.33 percent polymorphism. Molecular size of the amplicons ranged from 395 to 1308 bp and the PIC value was 0.86 (Table 4.15).

**4.3.2.1.10 OPF-5**

OPF-5 (Plate 4.10) RAPD marker produced a total of 14 loci, of which all 14 loci were polymorphic with 100 percent polymorphism. Molecular size of the amplicons ranged from 404 to 1400 bp and the PIC value 0.87 (Table 4.15).

**4.3.2.1.11 OPF-10**

RAPD profile obtained with primer OPF-10 (Plate 4.11) produced 12 loci, of which 11 loci were polymorphic with 91.66 percent polymorphism. Molecular size of the amplicons ranged from 451 to 1362 bp and the PIC value 0.87 (Table 4.15).

**4.3.2.2 Pooled RAPD data analysis**

The data collected from random amplification of polymorphic DNA, with 11 arbitrary primers produced a total of 1719 amplicons with 152 loci, of which 123 loci were polymorphic with an average of 80.01 percent polymorphism. On an average, 12 loci were generated per primer. Primers OPB-7, OPB-8 and OPB-12 have amplified maximum of 19, 16 and 16 loci out of which 18, 14 and 15 loci, respectively were found polymorphic, whereas minimum of 10 loci were amplified by primer OPD-2 out of which 8 were found polymorphic.

The highest polymorphism of 100% was exhibited by primer OPF-5 while lowest polymorphism of 35.71% was exhibited by primer OPD-16. Ronning *et al.* (1995); Bharad *et al.* (2009) and Guimaraes *et al.* (2013) obtained 100, 73 and 28.7% polymorphism, respectively. PIC value was ranged from minimum of 0.86



(OPD-2 and OPF-4) to maximum of 0.92 (OPB-7, OPB-10 and OPD-16) with an average of 0.89 indicating high levels of genetic diversity among different genotypes of *Annona* species.

Molecular size of the amplified PCR products ranged from 146 bp (OPB-7) to 1406 bp (OPB-6). Bharad *et al.*(2009) obtained PCR products of 208 bp to 1354 bp. RAPD primer OPD-16 has generated maximum number of 233 bands, whereas primer OPF-5 has produced minimum number of 104 bands. The details of amplified products are given in the table 4.20.

The highest similarity index value of 0.93 was found between ACC-2 and ACC-3, while the least similarity index value of 0.40 was found between *A. muricata* and ACC-4. The average similarity coefficient among genotypes was 0.70 (Appendix-I). Zhizang *et al.* (2011) obtained similarity coefficient ranged from 0.572 to 0.818.

#### **4.3.2.3 Clustering Pattern of different 20 *Annona* genotypes**

Clustering pattern of 20 *Annona* genotypes was produced by using the computer software “NTSYSpc version 2.02, based on UPGMA following the numerical taxonomic techniques and methods of Rholf (1998).

The pooled molecular data of 11 RAPD primers indicated that 5 clusters namely A to E were formed at a similarity coefficient of 0.70 (cut off value) (Fig 4.28). The dendrogram constructed using the pooled RAPD loci data showed that cluster A had *A. cherimola*, cluster B had *A. atemoya*, whereas cluster C included 16



genotypes (Red Sitaphal, Anand Selection, Sindhan, Balanagar, GJCA-1, Vidyanagar local, ACC-1, ACC-2, ACC-3, ACC-4, ACC-5, ACC-6, Sindhan×Anand Selection, Sindhan×Balanagar, Anand Selection ×Balanagar and Balanagar×Red Sitaphal). Cluster D and E possessed *A. reticulata* and *A. muricata*, respectively.

#### 4.3.2.4 Principle Component Analysis

The results of principle component analysis (PCA) carried out with 20 *Annona* genotypes (Fig 4.29 and 4.30) have coincided with the results of cluster analysis. The clustering patterns of 2D and 3D of PCA analysis were in accordance with the dendrogram clustering pattern which showed that genotypes viz., Red Sitaphal, Anand Selection, Sindhan, Balanagar, GJCA-1, Vidyanagar local, ACC-1, ACC-2, ACC-3, ACC-4, ACC-5, ACC-6, Sindhan × Anand Selection, Sindhan × Balanagar, Anand Selection × Balanagar and Balanagar × Red Sitaphal) are closely related and belonged to a single group of *A. squamosa*. It also revealed *Annona* species like *A. cherimola*, *A. reticulata*, *A. muricata* and *A. atemoya* are genetically far away related with themselves as well as with *A. squamosa*.

Ronning *et al.* (1995) Bharad *et al.* (2009) and Guimaraes *et al.* (2013) also demonstrated significant genetic diversity among different *Annona* genotypes using RAPD markers.







#### **4.3.2.5 Cophenetic correlation study (Mantel's test)**

Correlation study was carried out to compare the correlation of original similarity matrix of RAPD results with the dendrogram clustering pattern. Using the Cophmodule of NTSYSpc version 2.02, r value was calculated and results were also expressed graphically (Fig. 4.31). High correlation between the similarity matrix and dendrogram pattern was depicted by the r value which was found to be 0.96 which is good to fit.

#### **4.3.3 Microsatellites or Simple Sequence Repeat (SSR) marker analysis**

In addition to RAPD, Simple Sequence Repeat (SSR) primers were also used for genetic diversity analysis of twenty genotypes of *Annona* species. Different primers of LMCH series developed by Escibano *et al.* (2008) were used for SSR amplification in this investigation.

A total of 20 primers were used for preliminary screening, out of which, only 12 primers were found polymorphic (Table 4.16).

##### **4.3.3.1 Individual SSR primer profile analysis**

###### **4.3.3.1.1 LMCH-29**

PCR amplification with SSR primer LMCH-29 (Plate-12) produced a total of four alleles and all of them were polymorphic with 100 percent polymorphism. Total 25 bands were obtained and size of amplicons ranged from 196-280 bp. The marker showed PIC value of 0.496 (Table 4.16). Escibano *et al.* (2004) obtained 6 alleles with amplicon size of 185-195 bp in *A. cherimola*.

**4.3.3.1.2 LMCH-43**

The banding pattern generated by LMCH-43 (Plate 4.13) produced three alleles and all of them were polymorphic with 100 percent polymorphism. Total 21 bands were obtained and size of amplicons ranged from 210-375 bp. The marker showed PIC value of 0.177 (Table 4.16). Escribano *et al.* (2008) also obtained 3 alleles with amplicon size of 210-216 bp in *A. cherimola*.

**4.3.3.1.3 LMCH-48**

Two alleles (Plate 4.14) were generated by LMCH-48 SSR primer, of which only one was polymorphic with 50 percent polymorphism. Total 23 bands were obtained and size of amplicons ranged from 157-192 bp. The marker showed PIC value of 0.227 (Table 4.16). Escribano *et al.* (2008) obtained 5 alleles with amplicon size of 141-154 bp in *A. cherimola*.

**4.3.3.1.4 LMCH-70**

PCR amplification with SSR primer LMCH-70 (Plate 4.15) generated a total of three alleles and all of them were polymorphic with 100 percent polymorphism. Total 21 bands were obtained and size of amplicons ranged from 152-178bp. The marker showed PIC value of 0.254 (Table 4.16). Escribano *et al.* (2008) obtained 4 alleles with amplicon size of 135-148 bp in *A. cherimola*.

**4.3.3.1.5 LMCH-71**

The banding pattern generated by primer LMCH-71 (Plate 4.16) produced a total of three alleles, of which two were polymorphic with 66.67 percent

polymorphism. Total 22 bands were obtained and size of amplicons ranged from 282-497 bp. The marker showed PIC value of 0.169 (Table 4.16). Escribano *et al.* (2008) obtained 5 alleles with amplicon size of 272-290 bp in *A. cherimola*.

#### **4.3.3.1.6 LMCH-78**

Three alleles were generated by PCR amplification of LMCH-78 (Plate 4.17) and all of them were polymorphic with 100 percent polymorphism. Total 20 bands were obtained and size of amplicons ranged from 172-235 bp. The marker showed PIC value of 0.265 (Table 4.16). Escribano *et al.* (2008) obtained 2 alleles with amplicon size of 159-161 bp in *A. cherimola*.

#### **4.3.3.1.7 LMCH-79**

SSR profile generated by primer LMCH-79 (Plate 4.18) produced a total of five alleles and all of them were polymorphic with 100 percent polymorphism. Total 27 bands were obtained and size of amplicons ranged from 212-384 bp. The marker showed PIC value of 0.694 (Table 4.16). Escribano *et al.* (2008) obtained 3 alleles with amplicon size of 206-210 bp in *A. cherimola*.

#### **4.3.3.1.8 LMCH-112**

The banding pattern generated by SSR primer LMCH-112 (Plate 4.19) amplified a total of four alleles and all of them were polymorphic with 100 percent polymorphism. Total 23 bands were obtained and size of amplicons ranged from 194-278 bp. The marker showed PIC value of 0.525 (Table 4.16). Escribano *et al.* (2008) also obtained 4 alleles with amplicon size of 210-222bp in *A. cherimola*.

#### **4.3.3.1.9 LMCH-114**

PCR amplification with SSR primer LMCH-114 (Plate 4.20) amplified a total of three alleles and all of them were polymorphic with 100 percent polymorphism. Total 24 bands were obtained and size of amplicons ranged from 202-246 bp. The marker showed PIC value of 0.351 (Table 4.16). Escribano *et al.* (2008) also obtained 3 alleles with amplicon size of 203-215 bp in *A. cherimola*.

#### **4.3.3.1.10 LMCH-119**

Three alleles were generated by PCR amplification of primer LMCH-119 (Plate 4.21) and all of them were polymorphic with 100 percent polymorphism. Total 20 bands were obtained and size of amplicons ranged from 191-287 bp. The marker showed PIC value of 0.265 (Table 4.16). Escribano *et al.* (2008) obtained 4 alleles with amplicon size of 191-212 bp in *A. cherimola*.

#### **4.3.3.1.11 LMCH-122**

SSR profile generated by primer LMCH-122 (Plate 4.22) produced three alleles and all of them were polymorphic with 100 percent polymorphism. Total 23 bands were obtained and size of amplicons ranged from 190-212 bp. The marker showed PIC value of 0.310 (Table 4.16). This primer has not shown any amplification in *A. muricata*. Escribano *et al.* (2008) obtained 6 alleles with amplicons of 177-210 bp in *A. cherimola*.

#### 4.3.3.1.12 LMCH-128

The banding pattern generated by SSR primer LMCH-128 (Plate 4.23) amplified a total of three alleles and all of them were polymorphic with 100 percent polymorphism. Total 24 bands were obtained and size of amplicons ranged from 252-288 bp. The marker showed PIC value of 0.343 (Table 4.16). Escibano *et al.* (2008) obtained 6 alleles with amplicon size of 255-275 bp in *A. cherimola*.

Kwapata *et al.* (2007) and Escibano *et al.* (2008) also revealed high level of heterozygosity with 4 to 14 alleles per locus and 2-10 alleles per locus, respectively, in different genotypes of *A. cherimola*.

All above SSR primers showed cross species amplification in all the five species of *Annona viz.*, *A. cherimola*, *A. reticulata*, *A. muricata*, *A. atemoya* and *A. squamosa* (Escibano *et al.*, 2008), (except LMCH-122 in *A. muricata*) indicating that these primers can be useful for the study of genetic diversity and marker assisted breeding among these species.

#### 4.3.3.2 Pooled SSR analysis

SSR analysis of all twelve primers generated 39 alleles, of which 37 alleles were polymorphic with an average of 93.05 percent polymorphism. On an average, per primer 4 polymorphic alleles were generated. The highest polymorphism (100%) was exhibited by primers LMCH-29, LMCH-43, LMCH-70, LMCH-78, LMCH-79, LMCH-112, LMCH-114, LMCH-119, LMCH-122 and LMCH-128



while 66.67% polymorphism was exhibited by primer LMCH-71. Lowest percent polymorphism (50%) was exhibited by primer LMCH-48.

The SSR primers in the present investigation produced amplicons of different sizes. The molecular size of amplicons ranged from minimum of 152 bp (LMCH-70) to maximum of 497 bp (LMCH-71). Maximum numbers of 27 amplicons were generated by primer LMCH-79, whereas minimum numbers 20 amplicons were generated by primers LMCH-78 and LMCH-119. On an average, 23 amplicons were generated per primer. The polymorphic information content ranged from 0.169 (LMCH-71) to 0.694 (LMCH-79) with average PIC of 0.339 indicating high levels of genetic diversity among different genotypes of *Annona* species. The details of amplified products are given in the table 4.16.

The highest similarity index value of 1.00 was found between many *A. squamosa* genotypes and least similarity index value of 0.12 was found between all *A. reticulata* and *A. muricata*. The average similarity coefficient among genotypes was 0.67 (Appendix-II).

#### **4.3.3.3 Clustering Pattern of 20 *Annona* genotypes**

Clustering pattern in dendrogram generated by using the pooled molecular data of 12 SSR primers indicated that 20 *Annona* genotypes were clustered into five major groups (A to E) at an average cut of value 0.67 (Fig. 4.32).

Cluster A has been divided into two sub clusters viz., A1 and A2 which included *A. cherimola* and *A. atemoya*, respectively. Cluster B has divided into two



sub clusters B1 (Red Sitaphal, Sindhan, ACC-2 and ACC-4) and B2 (Balanagar, GJCA-1, ACC-1, ACC-2, ACC-3, ACC-4, ACC-5, ACC-6, Sindhan × Anand Selection, Sindhan × Balanagar, Anand Selection × Balanagar and Balanagar × Red Sitaphal). Cluster C included only single genotype (Vidyanagar local). Cluster D possessed *A. muricata*, whereas cluster E included *A. reticulata*.

#### 4.3.3.4 Principle Component Analysis

Grouping of genotypes were also evaluated by principle component analysis (PCA), which was performed by extracting Eigen value and Eigen vectors from a correlation matrix generated using standardized data matrix. The clustering patterns of 2D and 3D of PCA analysis (Fig 4.33 and 4.34) were in accordance with the dendrogram clustering pattern which showed that genotypes viz., Red Sitaphal, Sindhan, ACC-2 and ACC-4 are genetically similar and Anand selection, Balanagar, GJCA-1, ACC-1, ACC-3, ACC-5, ACC-6, Sindhan × Anand Selection, Sindhan × Balanagar, Anand Selection × Balanagar and Balanagar × Red Sitaphal are genetically close related, whereas genotype Vidyanagar local was genetically diverse from other genotypes of *A. squamosa*. It also revealed that *A. muricata* and *A. atemoya* are genetically diverse from other species like *A. cherimola*, *A. atemoya* and *A. squamosa*.

Kwapata *et al.* (2007) and Li lu *et al.* (2011) also demonstrated significant genetic diversity among *A. senegalensis* and pawpaw genotypes using SSR markers.







#### 4.3.3.5 Cophenetic correlation study (Mantel's test)

Correlation study was carried out to compare the correlation of original similarity matrix of SSR results with the dendrogram clustering pattern. Using the Coph module of NTSYSpc version 2.02, r value was calculated and results were also expressed graphically (Fig. 4.35). High correlation between the similarity matrix and dendrogram pattern was depicted by the r value which was found to be 0.98 which is good to fit.

#### 4.3.4 Comparison of RAPD and SSR primers

Mantel test was also employed to analyze the 'goodness of fit' for the UPGMA dendrogram generated by RAPD and SSR marker system. The Cophenetic correlation 'r' values obtained through Mantel's test are as follows;

|             | <b>RAPD</b> | <b>SSR</b> |
|-------------|-------------|------------|
| <b>RAPD</b> | 0.96        | 0.97       |
| <b>SSR</b>  | 0.97        | 0.98       |

Average Polymorphism for RAPD analysis was found to be 80.01%, whereas for SSR it was 93.05%. For RAPD analysis maximum band size was found to be 1406 bp and minimum was 146 bp, whereas in SSR maximum band size was 497 bp and minimum was 152 bp. Total numbers of bands in RAPD was 1719, whereas in SSR it was 272. PIC value for RAPD analysis ranged from 0.86 to 0.92, whereas for SSR analysis it ranged from 0.16 to 0.69. A Jaccard's similarity coefficient of all genotypes derived from RAPD data ranged from 0.40 to 0.93 with mean value 0.70, whereas for SSR data it ranged from 0.12 to 1.0 with mean value 0.67. The

cophenetic correlation value,  $r=0.96$  and  $r=0.98$  were studied for RAPD and SSR, respectively and found that both markers are good fit for cluster analysis.

This molecular study revealed that high genetic diversity exists between different *Annona* genotypes. RAPD markers were found more informative as compared to SSR for discriminating different *Annona* genotypes, therefore RAPD can be better exploited for identification of different genotypes of *Annona* species, which will be ultimately useful in the improvement of the crop.

## V. SUMMARY AND CONCLUSION

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*Annona* belongs to the family *Annonaceae* which is mostly known for its edible fruits. *Annona* fruits are of the world's best tasting fruits due to their sweet, delicious, creamy flesh and fragrant flavor when fully ripe. Fruits contain proteins, fatty acids, fibres, carbohydrates, vitamin C, antioxidants and minerals such as calcium, phosphorous and potassium. Leaves and seeds are potential source of secondary metabolites and antioxidants. Seeds contain good amount of oil which can be useful for industrial purpose.

The investigation entitled “**Morphological, biochemical and molecular characterization of different genotypes of *Annona* species**” was undertaken at different places like Morphological Characterization at Horticulture Farm, Department of Horticulture, Biochemical Characterization at Department of Biochemistry, Molecular Characterization at Department of Agricultural Biotechnology, Centre of Excellence in Biotechnology, AAU, Anand, during 2014-15.

The objectives of the present study were assessment of genetic variability in different genotypes of *Annona* species by morphological parameters, estimation of various biochemical parameters from fruits and seeds of *Annona* species and study the molecular polymorphism in different genotypes of *Annona* species through RAPD and SSR markers.

## **5.1 Materials and Methods**

Different materials and methods employed in the present investigation are very briefly summarized as follows;

### **5.1.1 Morphological characterization**

The experimental material comprised of 9 matured and fruiting genotypes collected from Horticulture farm, Department of Horticulture, BACA, AAU, Anand. Total 17 morphological characters were evaluated using completely randomized block design (CRBD) with three replications. Components of genetic variance were studied to know the existence of degree of genetic diversity and gene action. Correlation study was carried out for all the characters related to leaves, flowers, fruits and seeds.

### **5.1.2 Biochemical characterization**

The experimental materials comprised of nine matured and fruiting genotypes collected from Horticulture farm, Department of Horticulture, BACA, AAU, Anand. Total 13 biochemical parameters were evaluated for proximate fruit composition, antioxidant activity and determination of phytochemicals using samples grown by completely randomized block design (CRBD) with three replications.

Components of genetic variance were studied to know the existence of degree of genetic diversity and gene action. Correlation study was carried out to

understand the association among various quality parameters and also with fruit yield per plant.

### **5.1.3 Molecular characterization**

The experimental material comprised of 20 *Annona* genotypes, which were evaluated for genetic diversity at molecular level using 11 RAPD primers and 12 SSR primers.

## **5.2 Most salient results of the study**

### **5.2.1 Morphological characterization**

Study of morphological parameters revealed that *Annona* possess wide range of leaf length ranging from 5.14 cm (Red Sitaphal) to 21.38 cm (*A. atemoya*) with an average of 12.34 cm and leaf width ranging from 2.22 cm (Red Sitaphal) to 10.70 cm (*A. atemoya*) with an average of 5.01 cm.

Length of petals ranged from 2.35 cm (*A. reticulata*) to 4.35 cm (*A. atemoya*) with an average of 3.08 cm and width of petals ranged from 0.63 cm (Red Sitaphal) to 1.44 cm (*A. muricata*) with an average of 0.87 cm.

Fruit length varied from 6.87 cm (Red Sitaphal) to 10.95 cm (*A. muricata*) with an average of 8.87 cm and width of fruits ranged from 6.72 cm (Red Sitaphal) to 10.37 cm (*A. muricata*) with an average of 8.42 cm.

Fruit weight ranged from 87.89g (Red Sitaphal) to 370.41 g (*A. atemoya*) with an average of 191.76 g, volume of fruits ranged from 92 cm<sup>3</sup> (Red Sitaphal) to

340 cm<sup>3</sup> (*A. atemoya*) with an average of 182.66 cm<sup>3</sup> and density of the fruits ranged from 0.96 g/cm<sup>3</sup> (Red Sitaphal) to 1.13 g/cm<sup>3</sup> (*A. muricata*) with an average of 1.04 g/cm<sup>3</sup>.

Seed length ranged from 1.11 cm (Red Sitaphal) to 1.83 cm (*A. cherimoya*) with an average of 1.47 cm and width of seeds ranged from 0.52 cm (Red Sitaphal) to 0.88 cm (*A. atemoya*) with an average of 0.72 cm. Hundred seed weight was also recorded with the range from 12.67 g (Red Sitaphal) to 41.30 g (*A. atemoya*) and average of 25.06 g.

Number of fruits per plant ranged from 47 (*A. atemoya*) to 117 (*A. cherimoya*) with an average of 90 fruits and number of seeds per fruit ranged from 38 (Red Sitaphal) to 49 (Balanagar) with an average of 45 seeds per fruit.

Fruit yield was found to vary from 7.53 kg (Red Sitaphal) to 26.4 kg (*A. cherimola*) per plant with the average of 16.49 kg fruits per plant.

Variability component study revealed that leaf length, leaf width, petal length, petal width, fruit length, fruit width, fruit weight, fruit volume, number of fruits per plant, seed length, seed width, number of seeds per fruit and hundred seed weight possessed high heritability coupled with high genetic advance.

Correlation study between different morphological parameters suggested the strong genotypic and phenotypic association of fruit yield per plant with leaf length, leaf width, fruit length, fruit width, fruit weight, fruit volume and number of fruits

per plant so these traits would be given due weightage for direct improvement of fruit yield in *Annona* species.

### 5.2.2 Biochemical characterization

Different biochemical components were measured from fruit pulp and seeds of *Annona*. Proximate analysis revealed that 100g of pulp contain 72.55-81.23% moisture content, 1.32-1.69% ash content, 16.48-24.05% total carbohydrates, 8.92-17.44% total soluble sugars, 3.27-8.39% reducing sugars, 1.14-2.14% protein content, 2.18-3.82% fibre content, 0.26-0.50% phenol content, 0.17-0.65% titratable acidity and 19.6-39.24% ascorbic acid content, whereas seed contained 23.13-32.50% of oil content in different genotypes of *Annona* species.

Study of antioxidant activity reported that *Annona* contained good amount of antioxidant activity in their seeds and pulps. *Annona* seeds possessed antioxidant activity of 0.29-0.48% which was comparatively higher than the antioxidant activity of pulp which varied from 0.19-0.31% in different genotypes. *A. muricata* showed maximum antioxidant activity as compared to other species of *Annona*.

Phytochemical screening study revealed that *Annona* contain many secondary metabolites like alkaloids, flavenoids, steroids, terpenoids and phenolic compounds, whereas saponin, glycosides were found absent. Generally, seeds of *Annona* were found to possess higher amount of phytochemicals as compared to pulp. Genotypes like *A. muricata*, *A. cherimola*, *A. atemoya* and *A. squamosa*

showed the more amount of above secondary metabolites whereas few compounds were found absent in *A. reticulata*.

Correlation study between different biochemical parameters suggested the strong genotypic and phenotypic association of total carbohydrates with TSS, reducing sugars and fibre content and ascorbic acid with antioxidant activity, which means quality improvement in *Annona* species for simultaneous two or more parameters can be possible.

### 5.2.3 Molecular characterization

Germplasm characterization is an important link between the conservation and utilization of plant genetic resources. A total of 20 genotypes of *Annona* species were characterized through RAPD and SSR markers.

#### (i) RAPD analysis

11 RAPD primers produced a total of 1719 amplicons with 152 loci, of which 123 loci were polymorphic. Polymorphism was ranged from 35.71% (OPD-16) to 100% (OPF-5) with average of 80.1%, whereas PIC value ranged from 0.86 (OPD-2 & OPF-4) to 0.92 (OPB-7, OPB-8 & OPD-16) with average of 0.89. Molecular weight of the PCR products ranged from 146 to 1406 bp.

RAPD primers based cluster analysis has produced 5 clusters indicating that five *Annona* species are genetically diverse. Other 16 genotypes have grouped into one cluster indicating that they belong to a single species, *A. squamosa*. Similarity

coefficient ranged from 0.40 to 0.93. RAPD primers showed ‘r’ value of 0.96 indicating good to fit for genetic diversity analysis of *Annona* species.

## **(ii) SSR analysis**

SSR analysis of 12 primers generated 39 alleles, of which 37 alleles were polymorphic with an average of 93.05 percent polymorphism. 100% polymorphism was exhibited by primers LMCH-29, LMCH-43, LMCH-70, LMCH-78, LMCH-79, LMCH-112, LMCH-114, LMCH-119, LMCH-122 and LMCH-128, whereas 50% was exhibited by primer LMCH-48. PIC content ranged from 0.169 (LMCH-71) to 0.694 (LMCH-79) with average PIC of 0.339.

Dendrogram using SSR primers showed five clusters indicating the existence of genetic diversity among all five *Annona* species and genotypes of *A. squamosa*. Cluster A contained A1 (*A. cherimola*) and A2 (*A. atemoya*) indicating that they share some common genetic base. Cluster B contained B1 (Red Sitaphal, Sindhan, ACC-2 and ACC-4) and B2 (Balanagar, GJCA-1, ACC-1, ACC-2, ACC-3, ACC-4, ACC-5, ACC-6, Sindhan × Anand Selection, Sindhan × Balanagar, Anand Selection × Balanagar and Balanagar × Red Sitaphal), whereas Cluster C contained only Vidyanagar local. Cluster D included *A. muricata*, while *A. reticulata* was present in cluster E. Similarity index value ranged from 0.12 to 1.00. SSR primers showed ‘r’ value of 0.98 indicating good to fit for genetic diversity analysis of *Annona* species.

The present study on “**Morphological, biochemical and molecular characterization of different genotypes of *Annona* species**” can be concluded as follows;

- ❖ Among all the *Annona* genotypes, *A. atemoya* possessed significantly high mean value of leaf length, leaf width, petiole length, petal length, petal width, fruit width, fruit weight and fruit volume, whereas *A. cherimola* possessed for higher number of fruits per plant. These two genotypes can be exploited in the improvement of *Annona* species for higher yield.
- ❖ Variability component study revealed that leaf length, leaf width, petal length, petal width, fruit length, fruit width, fruit weight, fruit volume, number of fruits per plant, seed length, seed width, number of seeds per fruit and hundred seed weight possessed high heritability coupled with high genetic advance, which indicated that these characters are mainly governed by additive gene action, hence simple selection will be very effective for crop improvement.
- ❖ Correlation study between different morphological parameters suggested the strong genotypic and phenotypic association of fruit yield per plant with leaf length, leaf width, fruit length, fruit width, fruit weight, fruit volume and number of fruits per plant so these traits would be given due weightage for direct improvement of fruit yield in *Annona* species.

- ❖ *A. muricata* contained significantly higher amount of seed oil, antioxidant activity (seeds and pulp) and various phytochemicals, indicating a potential scope for fuel industries and pharmaceutical industries, respectively.
- ❖ *A. Squamosa* var. GJCA-1, Balanagar and Sindhan possessed significantly higher amount of carbohydrates, TSS, proteins, edible fibres and ascorbic acid, suggesting a good choice for fruit breeders and consumers.
- ❖ Correlation study between different biochemical parameters suggested the strong genotypic and phenotypic association of total carbohydrates with TSS, reducing sugars and fibre content and ascorbic acid with antioxidant activity, which means quality improvement in *Annona* species for simultaneous two or more parameters can be possible.
- ❖ Molecular study revealed the existence of enough genetic diversity amongst different genotypes of *Annona* species.
- ❖ RAPD markers were found more informative as compared to SSR for discriminating different *Annona* genotypes; therefore RAPD can be exploited for identification of different genotypes of *Annona* species, which will be useful in the improvement of the crop.

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**Appendix-I. Jaccard's similarity coefficient based on RAPD data of 20 *Annona* genotypes**

|    | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   | 13   | 14   | 15   | 16   | 17   | 18   | 19   | 20 |
|----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|----|
| 1  | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 2  | 0.50 | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 3  | 0.42 | 0.45 | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 4  | 0.68 | 0.49 | 0.53 | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 5  | 0.63 | 0.50 | 0.42 | 0.65 | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 6  | 0.57 | 0.50 | 0.45 | 0.58 | 0.83 | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 7  | 0.59 | 0.45 | 0.42 | 0.56 | 0.78 | 0.81 | 1    |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 8  | 0.64 | 0.52 | 0.45 | 0.60 | 0.75 | 0.75 | 0.81 | 1    |      |      |      |      |      |      |      |      |      |      |      |    |
| 9  | 0.68 | 0.50 | 0.45 | 0.65 | 0.80 | 0.79 | 0.74 | 0.81 | 1    |      |      |      |      |      |      |      |      |      |      |    |
| 10 | 0.62 | 0.49 | 0.44 | 0.64 | 0.79 | 0.75 | 0.67 | 0.70 | 0.83 | 1    |      |      |      |      |      |      |      |      |      |    |
| 11 | 0.65 | 0.56 | 0.46 | 0.66 | 0.88 | 0.81 | 0.72 | 0.79 | 0.87 | 0.83 | 1    |      |      |      |      |      |      |      |      |    |
| 12 | 0.61 | 0.59 | 0.45 | 0.61 | 0.78 | 0.77 | 0.71 | 0.77 | 0.75 | 0.74 | 0.84 | 1    |      |      |      |      |      |      |      |    |
| 13 | 0.64 | 0.58 | 0.45 | 0.64 | 0.81 | 0.76 | 0.72 | 0.78 | 0.77 | 0.76 | 0.89 | 0.93 | 1    |      |      |      |      |      |      |    |
| 14 | 0.67 | 0.49 | 0.40 | 0.59 | 0.77 | 0.76 | 0.73 | 0.80 | 0.82 | 0.76 | 0.78 | 0.78 | 0.77 | 1    |      |      |      |      |      |    |
| 15 | 0.67 | 0.53 | 0.45 | 0.64 | 0.84 | 0.81 | 0.76 | 0.85 | 0.84 | 0.77 | 0.86 | 0.82 | 0.83 | 0.88 | 1    |      |      |      |      |    |
| 16 | 0.60 | 0.54 | 0.45 | 0.65 | 0.83 | 0.80 | 0.70 | 0.77 | 0.74 | 0.77 | 0.85 | 0.91 | 0.88 | 0.77 | 0.83 | 1    |      |      |      |    |
| 17 | 0.58 | 0.53 | 0.45 | 0.61 | 0.76 | 0.75 | 0.65 | 0.68 | 0.69 | 0.70 | 0.78 | 0.81 | 0.79 | 0.70 | 0.74 | 0.84 | 1    |      |      |    |
| 18 | 0.61 | 0.52 | 0.44 | 0.62 | 0.84 | 0.79 | 0.76 | 0.79 | 0.78 | 0.79 | 0.82 | 0.84 | 0.81 | 0.86 | 0.88 | 0.87 | 0.75 | 1    |      |    |
| 19 | 0.61 | 0.54 | 0.44 | 0.62 | 0.86 | 0.77 | 0.70 | 0.73 | 0.74 | 0.75 | 0.84 | 0.86 | 0.81 | 0.77 | 0.80 | 0.90 | 0.83 | 0.86 | 1    |    |
| 20 | 0.67 | 0.53 | 0.45 | 0.64 | 0.80 | 0.79 | 0.78 | 0.87 | 0.84 | 0.75 | 0.82 | 0.80 | 0.81 | 0.82 | 0.86 | 0.81 | 0.73 | 0.84 | 0.78 | 1  |

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|----------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 6.    | Anand selection  | 11.   | ACC-1    | 16.   | ACC-6                     |
| 2.    | <i>Annona reticulata</i> | 7.    | Sindhan          | 12.   | ACC-2    | 17.   | Sindhan×Anand selection   |
| 3.    | <i>Annona muricata</i>   | 8.    | Balanagar        | 13.   | ACC-3    | 18.   | Sindhan×Balanagar         |
| 4.    | <i>Annona atemoya</i>    | 9.    | GJCA-1           | 14.   | ACC-4    | 19.   | Anand selection×Balanagar |
| 5.    | Red sitaphal             | 10.   | Vidyanagar local | 15.   | ACC-5    | 20.   | Balanagar×Red sitaphal    |

**Appendix-II. Jaccard's similarity coefficient based on SSR data of 20 *Annona* genotypes**

|    | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   | 13   | 14   | 15   | 16   | 17   | 18   | 19   | 20 |
|----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|----|
| 1  | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 2  | 0.29 | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 3  | 0.39 | 0.12 | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 4  | 0.69 | 0.25 | 0.37 | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 5  | 0.50 | 0.21 | 0.47 | 0.45 | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 6  | 0.45 | 0.22 | 0.33 | 0.37 | 0.79 | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 7  | 0.50 | 0.21 | 0.47 | 0.45 | 1.00 | 0.79 | 1    |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 8  | 0.43 | 0.21 | 0.32 | 0.35 | 0.73 | 0.92 | 0.73 | 1    |      |      |      |      |      |      |      |      |      |      |      |    |
| 9  | 0.45 | 0.22 | 0.33 | 0.37 | 0.79 | 1.00 | 0.79 | 0.92 | 1    |      |      |      |      |      |      |      |      |      |      |    |
| 10 | 0.61 | 0.18 | 0.53 | 0.59 | 0.76 | 0.61 | 0.76 | 0.58 | 0.61 | 1    |      |      |      |      |      |      |      |      |      |    |
| 11 | 0.45 | 0.22 | 0.33 | 0.37 | 0.79 | 1.00 | 0.79 | 0.92 | 1.00 | 0.61 | 1    |      |      |      |      |      |      |      |      |    |
| 12 | 0.48 | 0.20 | 0.44 | 0.48 | 0.93 | 0.73 | 0.93 | 0.69 | 0.73 | 0.82 | 0.73 | 1    |      |      |      |      |      |      |      |    |
| 13 | 0.45 | 0.22 | 0.33 | 0.37 | 0.79 | 1.00 | 0.79 | 0.92 | 1.00 | 0.61 | 1.00 | 0.73 | 1    |      |      |      |      |      |      |    |
| 14 | 0.57 | 0.21 | 0.39 | 0.45 | 0.86 | 0.79 | 0.86 | 0.73 | 0.79 | 0.67 | 0.79 | 0.80 | 0.79 | 1    |      |      |      |      |      |    |
| 15 | 0.50 | 0.21 | 0.32 | 0.40 | 0.73 | 0.92 | 0.73 | 0.86 | 0.92 | 0.58 | 0.92 | 0.69 | 0.92 | 0.86 | 1    |      |      |      |      |    |
| 16 | 0.45 | 0.22 | 0.33 | 0.37 | 0.79 | 1.00 | 0.79 | 0.92 | 1.00 | 0.61 | 1.00 | 0.73 | 1.00 | 0.79 | 0.92 | 1    |      |      |      |    |
| 17 | 0.45 | 0.22 | 0.33 | 0.37 | 0.79 | 1.00 | 0.79 | 0.92 | 1.00 | 0.61 | 1.00 | 0.73 | 1.00 | 0.79 | 0.92 | 1.00 | 1    |      |      |    |
| 18 | 0.45 | 0.22 | 0.33 | 0.37 | 0.79 | 1.00 | 0.79 | 0.92 | 1.00 | 0.61 | 1.00 | 0.73 | 1.00 | 0.79 | 0.92 | 1.00 | 1.00 | 1    |      |    |
| 19 | 0.45 | 0.22 | 0.33 | 0.37 | 0.79 | 1.00 | 0.79 | 0.92 | 1.00 | 0.61 | 1.00 | 0.73 | 1.00 | 0.79 | 0.92 | 1.00 | 1.00 | 1.00 | 1    |    |
| 20 | 0.45 | 0.22 | 0.33 | 0.37 | 0.79 | 1.00 | 0.79 | 0.92 | 1.00 | 0.61 | 1.00 | 0.73 | 1.00 | 0.79 | 0.92 | 1.00 | 1.00 | 1.00 | 1.00 | 1  |

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|----------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 6.    | Anand selection  | 11.   | ACC-1    | 16.   | ACC-6                     |
| 2.    | <i>Annona reticulata</i> | 7.    | Sindhan          | 12.   | ACC-2    | 17.   | Sindhan×Anand selection   |
| 3.    | <i>Annona muricata</i>   | 8.    | Balanagar        | 13.   | ACC-3    | 18.   | Sindhan×Balanagar         |
| 4.    | <i>Annona atemoya</i>    | 9.    | GJCA-1           | 14.   | ACC-4    | 19.   | Anand selection×Balanagar |
| 5.    | Red sitaphal             | 10.   | Vidyanagar local | 15.   | ACC-5    | 20.   | Balanagar×Red sitaphal    |

|                                                                                   |       |                 |
|-----------------------------------------------------------------------------------|-------|-----------------|
|  | ..... | <i>Annexure</i> |
|-----------------------------------------------------------------------------------|-------|-----------------|

**Appendix-III**  
**Weekly meteorological data recorded during the crop season**  
**(July-2014 to Oct-2014)**

**Season: 2014-15**  
**Zone: Central zone**  
**State: Gujarat**  
**Location: Anand**

**Latitude: North 22°35'**  
**Longitude: East 72°55'**  
**Altitude: 45m above MSL**  
**Soil type: Sandy loam**

| Month | Std. week        | Date                                           | Temperature (°C) |      | Humidity (%) | Rainfall (mm) |
|-------|------------------|------------------------------------------------|------------------|------|--------------|---------------|
|       |                  |                                                | Max.             | Min. |              |               |
| 1     | 2                | 3                                              | 4                | 5    | 6            | 7             |
| July  | 28 <sup>th</sup> | 12 <sup>th</sup> July - 18 <sup>th</sup> July  | 37.2             | 27.6 | 70           | 41.0          |
|       | 29 <sup>th</sup> | 19 <sup>th</sup> July - 25 <sup>th</sup> July  | 33.2             | 25.3 | 85           | 150.4         |
|       | 30 <sup>th</sup> | 26 <sup>th</sup> July - 01 <sup>th</sup> Aug.  | 30.5             | 25.3 | 94           | 217.8         |
| Aug.  | 31 <sup>st</sup> | 02 <sup>th</sup> Aug. - 08 <sup>th</sup> Aug.  | 31.0             | 25.2 | 91           | 81.6          |
|       | 32 <sup>th</sup> | 09 <sup>th</sup> Aug. - 15 <sup>th</sup> Aug.  | 31.6             | 25.1 | 87           | 61.8          |
|       | 33 <sup>th</sup> | 16 <sup>th</sup> Aug. - 22 <sup>th</sup> Aug.  | 33.1             | 24.7 | 84           | 10.6          |
|       | 34 <sup>th</sup> | 23 <sup>rd</sup> Aug. - 29 <sup>th</sup> Aug.  | 34.4             | 25.7 | 78           | 8.6           |
|       | 35 <sup>th</sup> | 30 <sup>th</sup> Aug. - 05 <sup>th</sup> Sept. | 33.0             | 24.0 | 85           | 61.0          |
| Sept. | 36 <sup>th</sup> | 06 <sup>th</sup> Sept.-12 <sup>th</sup> Sept.  | 30.9             | 24.5 | 91           | 245.2         |
|       | 37 <sup>th</sup> | 13 <sup>th</sup> Sept.-19 <sup>th</sup> Sept.  | 29.9             | 26.0 | 90           | 47.2          |
|       | 38 <sup>th</sup> | 20 <sup>th</sup> Sept.-26 <sup>th</sup> Sept.  | 32.8             | 26.2 | 81           | 36.8          |
|       | 39 <sup>th</sup> | 27 <sup>th</sup> Sept - 03 <sup>rd</sup> Oct.  | 35.1             | 26.8 | 74           | 0.0           |
| Oct.  | 40 <sup>th</sup> | 04 <sup>th</sup> Oct. - 10 <sup>th</sup> Oct.  | 36.9             | 24.1 | 66           | 0.8           |
|       | 41 <sup>th</sup> | 11 <sup>th</sup> Oct. - 17 <sup>th</sup> Oct.  | 37.3             | 21.4 | 66           | 0.0           |
|       | 42 <sup>th</sup> | 18 <sup>th</sup> Oct. - 24 <sup>th</sup> Oct.  | 37.0             | 19.2 | 63           | 0.0           |
|       | 43 <sup>rd</sup> | 25 <sup>th</sup> Oct. -31 <sup>th</sup> Oct.   | 36.0             | 18.8 | 66           | 0.0           |

**Source:** Meteorological Observatory, B.A.C.A., A.A.U., Anand, Gujarat.



**Fig 3.1 OVERVIEW OF ANNONA ORCHARD AT HORTICULTURE FARM, BACA, AAU, ANAND**

**Fig 4.1 Morphological variations in leaves of different genotypes of *Annona* species**

**A) Variation in leaf morphology among different species of *Annona***



**(1) *Annona cherimola* Mill.**



**(2) *Annona reticulata* L.**



**(3) *Annona muricata* L**



**(4) *Annona atemoya* Mabb.**



**(5) *Annona squamosa* L.**

**B) Variation in leaf morphology among different varieties of *Annona squamosa***



**(1) *Annona squamosa* L.  
(Variety- Red sitaphal)**



**(2) *Annona squamosa* L.  
(Variety- Anand Selection)**



**(3) *Annona squamosa* L.  
(Variety- Sindhan)**



**(4) *Annona squamosa* L.  
(Variety- Balanagar)**



**(5) *Annona squamosa* L.  
(Variety- GJCA-1)**

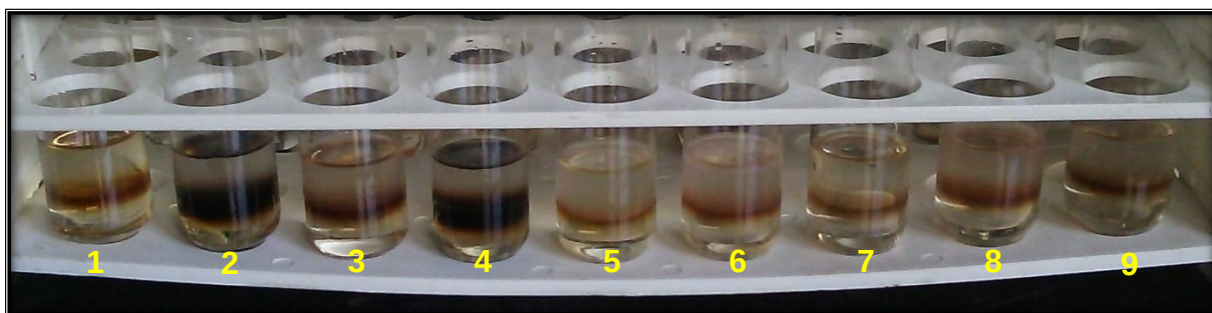


**Fig 4.24** Phytochemical screening for steroids in seeds of different *Annona* genotypes

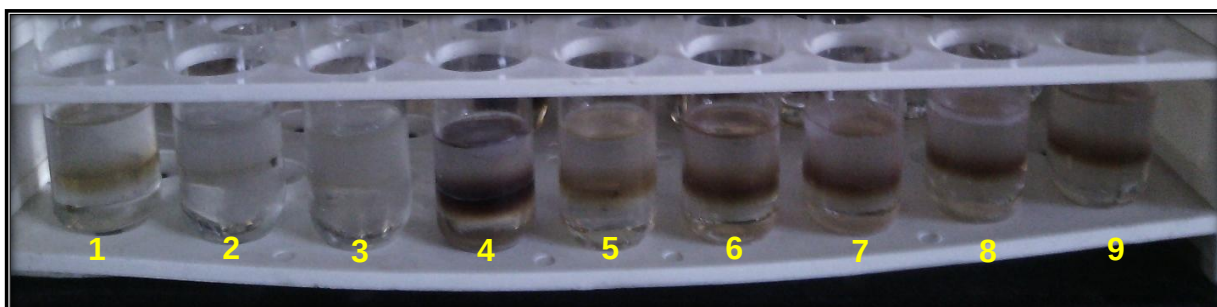


**Fig 4.25** Phytochemical screening for terpenoids in pulps of different *Annona* genotypes

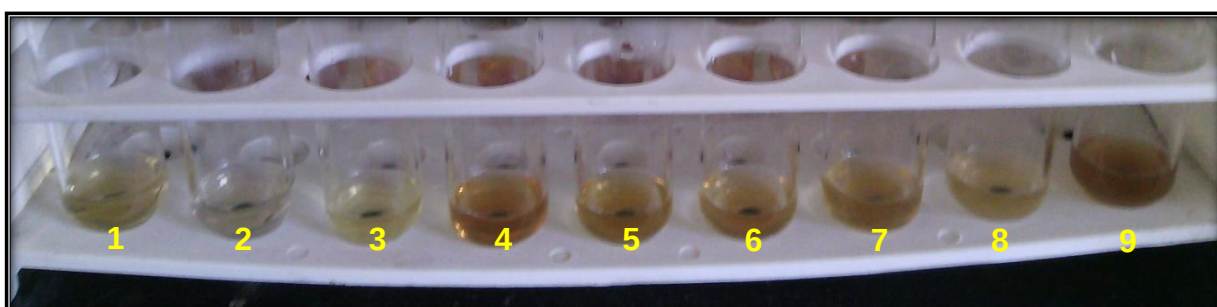


**Fig.4.26** Phytochemical screening for alkaloids in seeds of different *Annona* genotypes

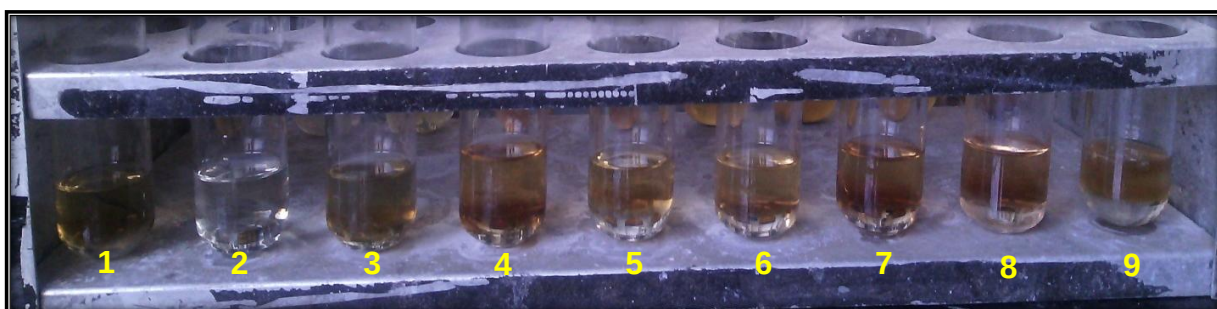


**Fig.4.27** Phytochemical screening for tannins in pulps of different *Annona* genotypes

1. *A. cherimola*, 2. *A. reticulata*, 3. *A. atemoya*, 4. *A. muricata*, 5. Red sitaphal,
6. Anand Selection, 7. Sindhan, 8. Balanagar, 9. GJCA-1.

**Fig 4.4 Morphological variations in flowers of different genotypes of *Annona* species**

**A) Variation in flower morphology among different species of *Annona***



**(1) *Annona cherimola* Mill.**



**(2) *Annona reticulata* L.**



**(3) *Annona atemoya* Mabb.**



**(4) *Annona squamosa* L.**

**B) Variation in flower morphology among different varieties of *Annona squamosa***



**(1) *A. squamosa* L. (Variety- Red Sitaphal)**



**(2) *A. squamosa* L. (Variety- Anand Selection)**



**(3) *A. squamosa* L. (Variety- Sindhan)**



**(4) *A. squamosa* L. (Variety- Balanagar)**



**(5) *A. squamosa* L. (Variety- GJCA-1)**

**Fig 4.5 Morphological variations in fruits of different genotypes of *Annona* species**

**A) Variation in fruit morphology among different species of *Annona***



**(1) *Annona cherimola* Mill. (2n=14)**



**(2) *Annona reticulata* L. (2n=14)**



**(3) *Annona muricata* L. (2n=14)**

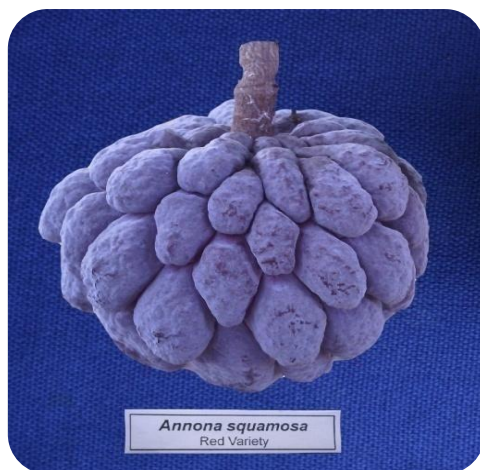


**(4) *Annona atemoya* Mabb. (2n=14)**



**(5) *Annona squamosa* L. (2n=14)**

**B) Variation in fruit morphology among different varieties of *Annona squamosa***



**(1) Red Sitaphal**



**(2) Ananand Selection**



**(3) Sindhan**



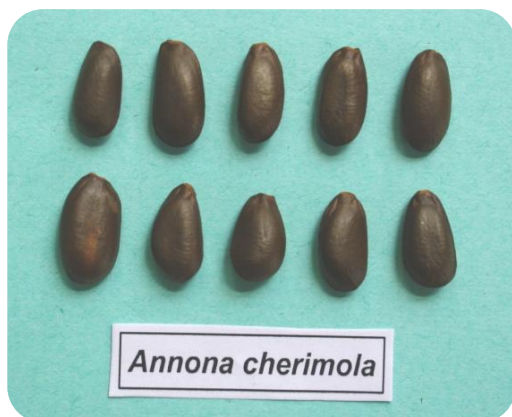
**(4) Balanagar**



**(5) GJCA-1**

**Fig 4.8 Morphological variations in seeds of different of *Annona* species**

**A) Variation in seed morphology among different *Annona* species**



**(1) *Annona cherimola* Mill.**



**(2) *Annona reticulata* L.**



**(3) *Annona muricata* L.**



**(4) *Annona atemoya* Mabb.**



**(5) *Annona squamosa* L.**

**B) Variation in seed morphology among different varieties of *Annona squamosa***



**(1) *Annona squamosa* L.  
(Variety- Red sitaphal)**



**(2) *Annona squamosa* L.  
(Variety- Anand Selection)**



**(3) *Annona squamosa* L.  
(Variety- Sindhan)**



**(4) *Annona squamosa* L.  
(Variety- Balanagar)**



**(5) *Annona squamosa* L.  
(Variety- GJCA-1)**

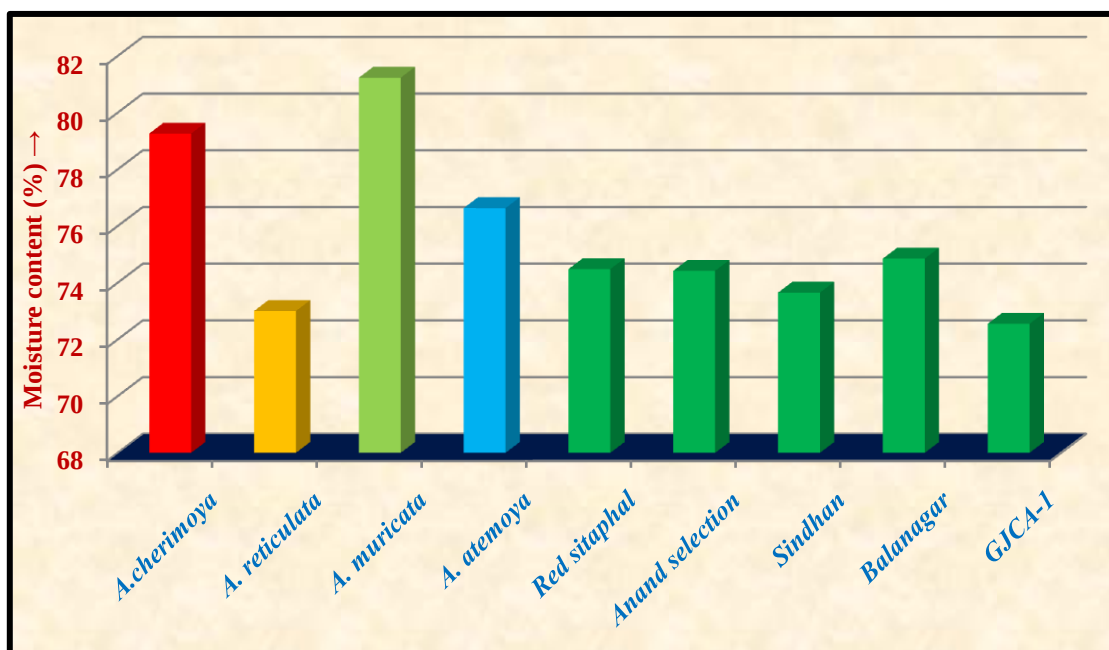


Fig 4.11 Moisture content (%) per 100g of fruit pulp of different *Annona* genotypes

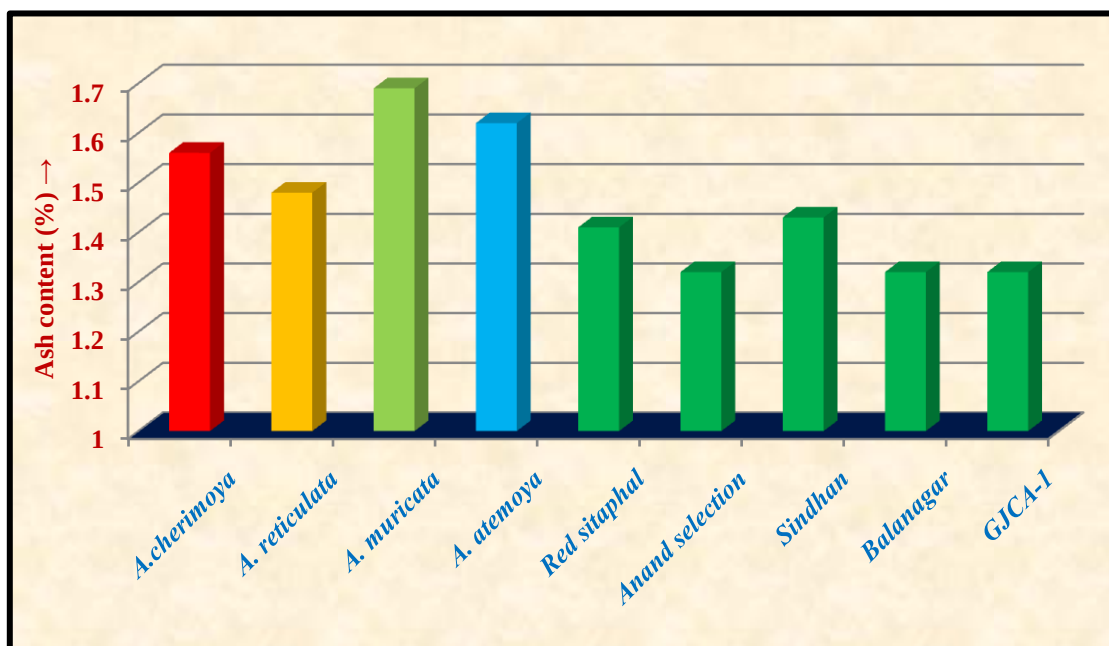


Fig 4.12 Ash content (%) per 100g of fruit pulp of different *Annona* genotypes

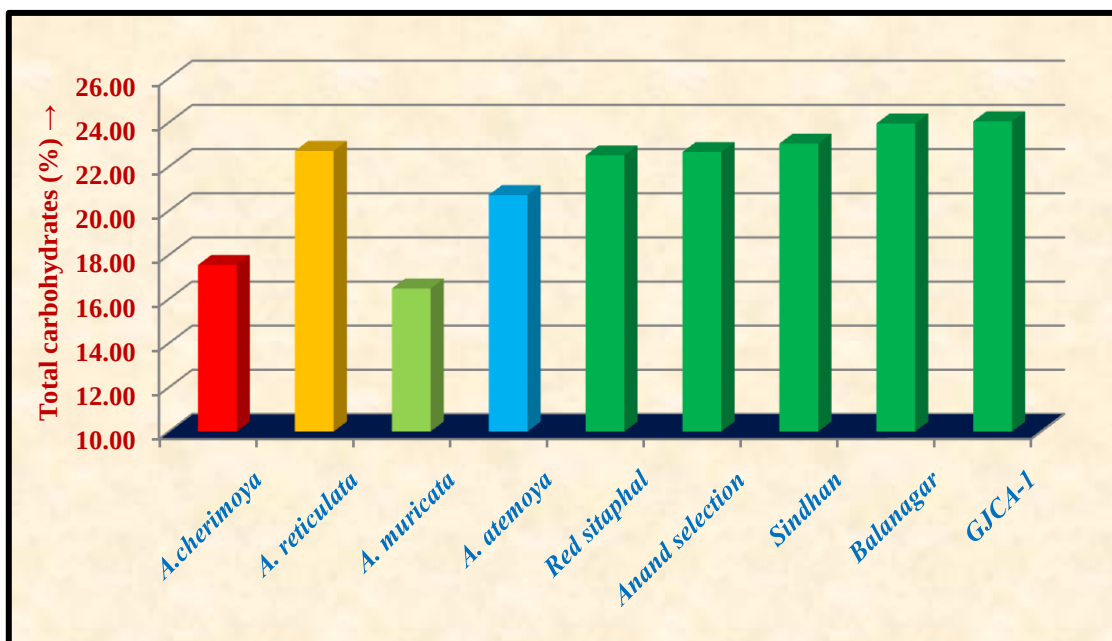


Fig 4.13 Total Carbohydrates (%) per 100g of fruit pulp of different *Annona* genotypes

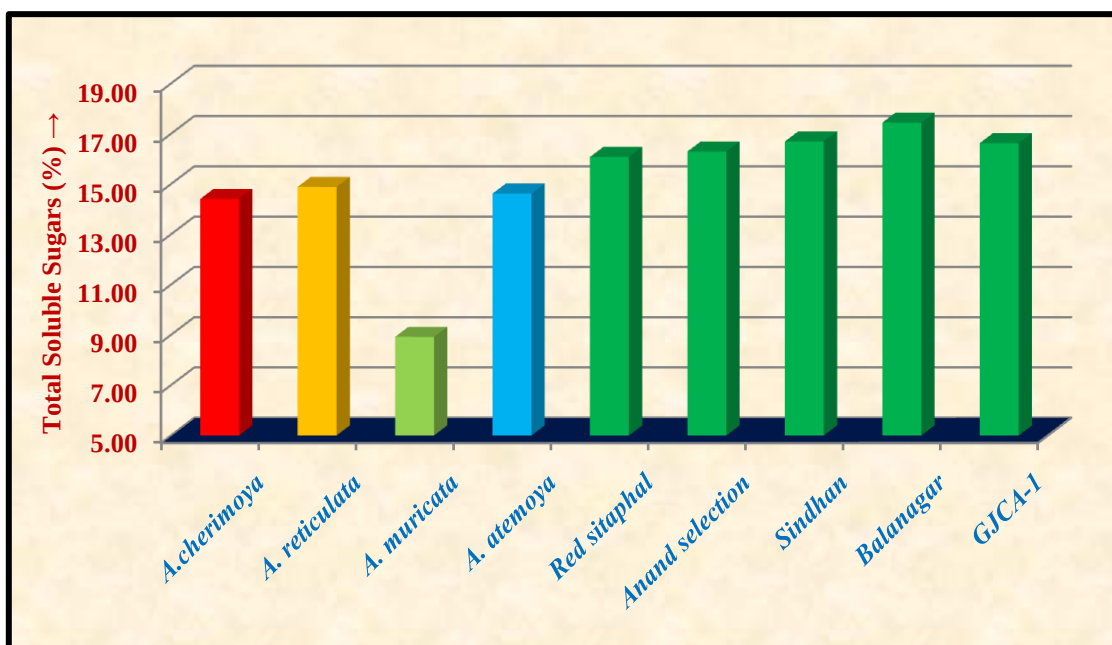


Fig 4.14 TSS content (%) per 100g of fruit pulp of different *Annona* genotypes

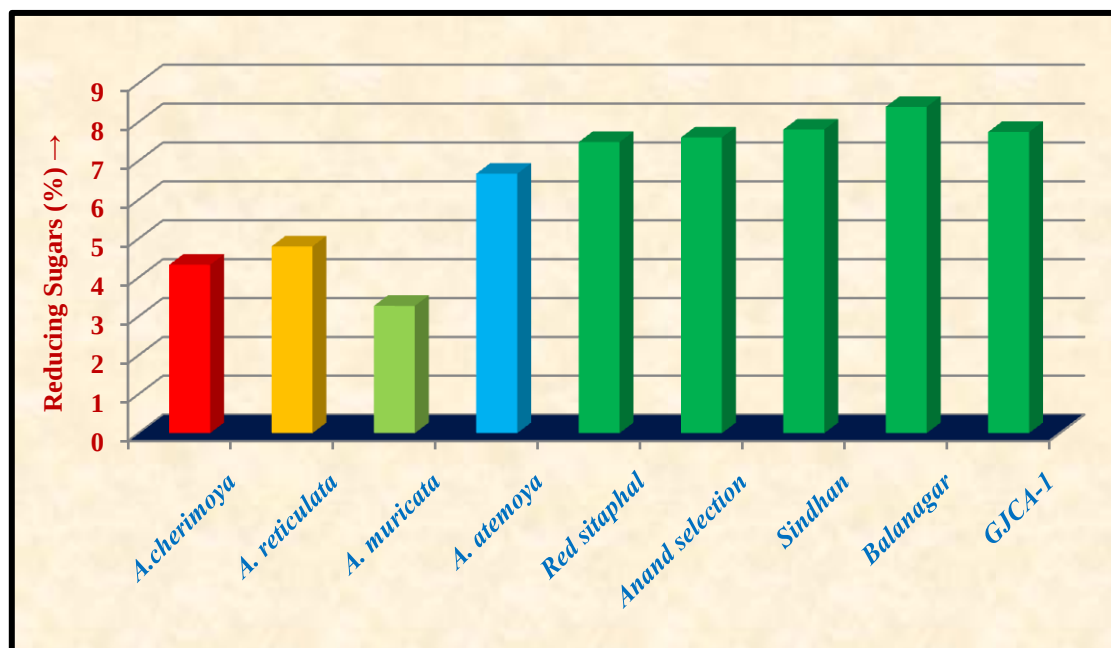


**Fig 4.15 Reducing Sugars (%) per 100g of fruit pulp of different *Annona* genotypes**

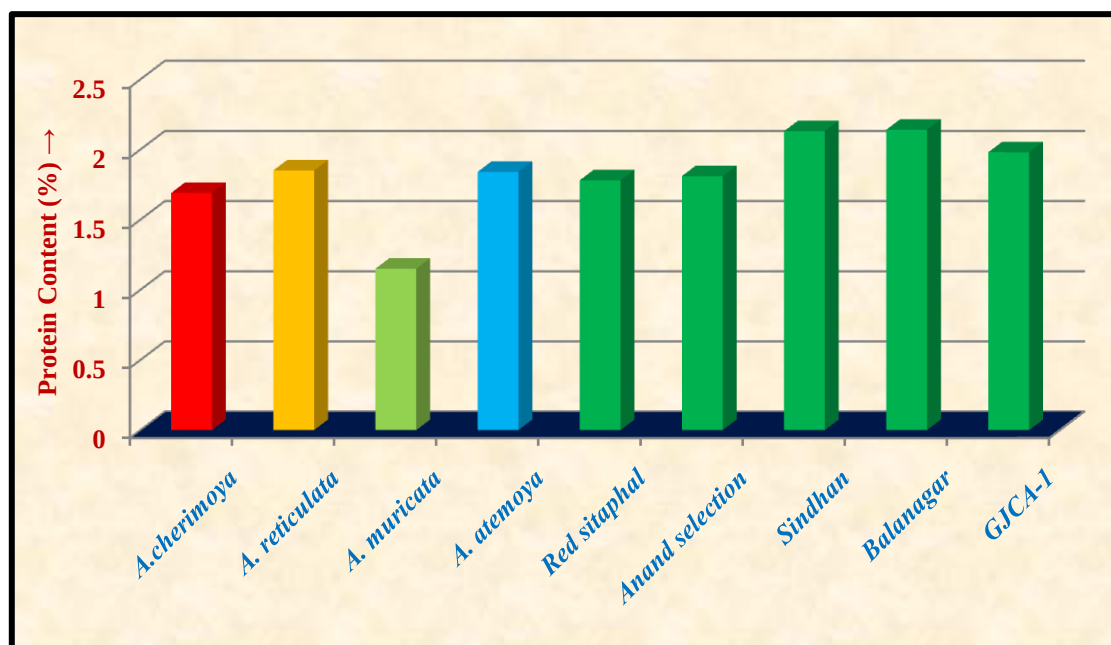


**Fig 4.16 Protein content (%) per 100g of fruit pulp of different *Annona* genotypes**

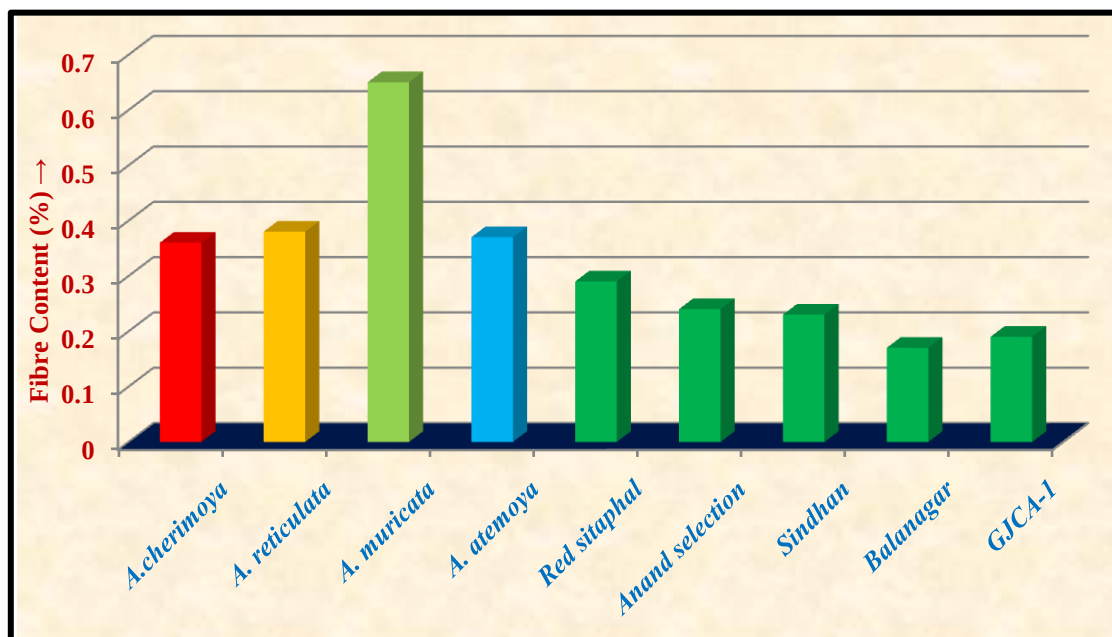


Fig 4.17 Fibre content (%) per 100g of fruit pulp of different *Annona* genotypes

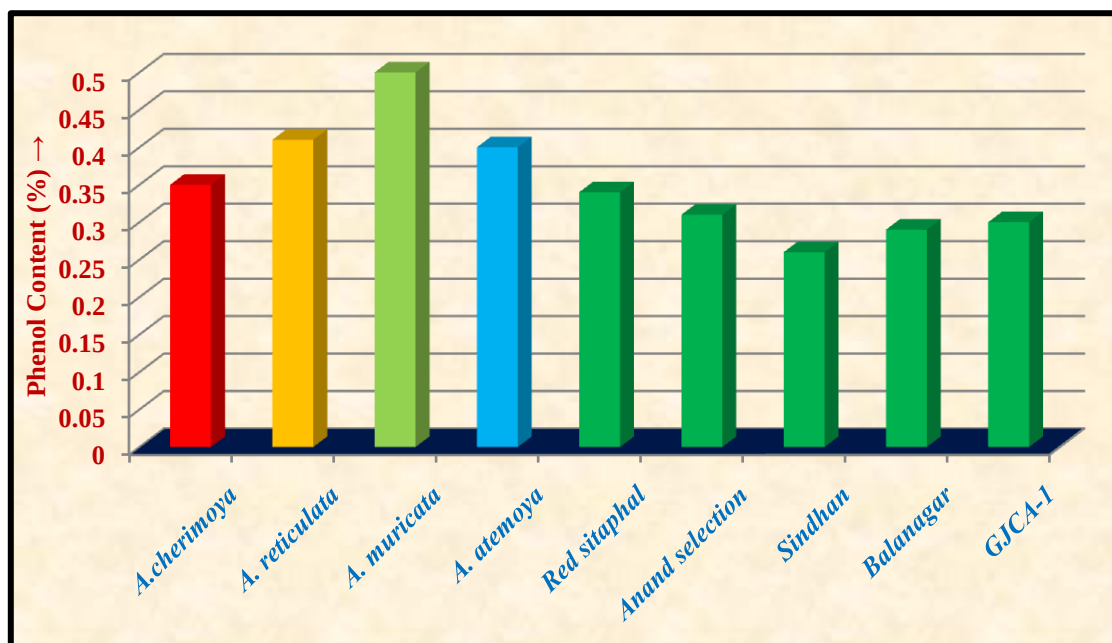


Fig 4.18 Phenol content (%) per 100g of fruit pulp of different *Annona* genotypes

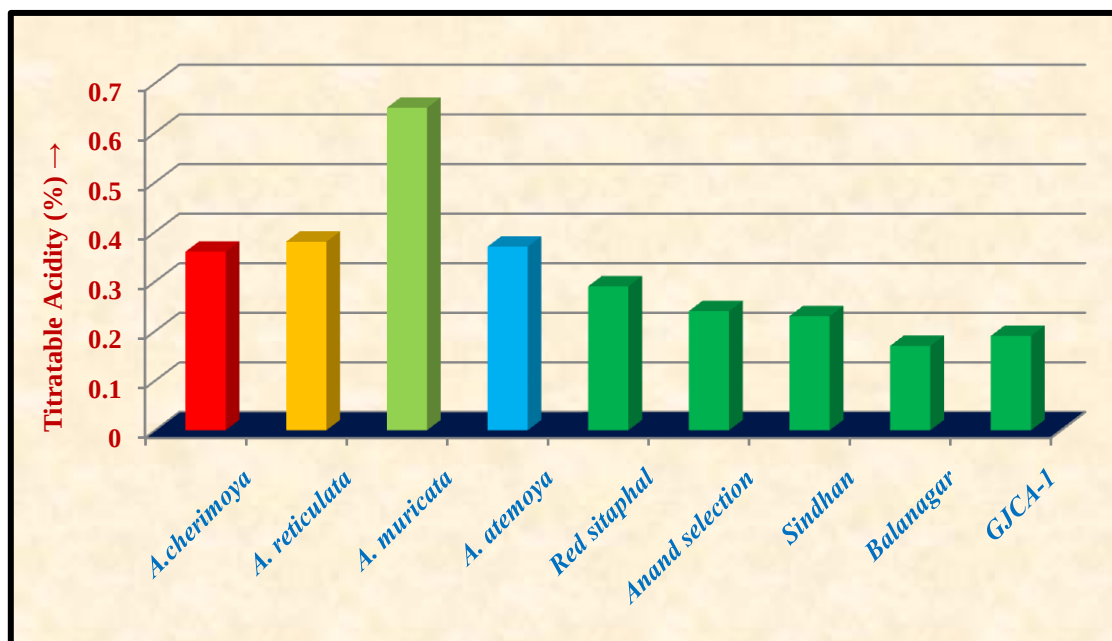


Fig 4.19 Titratable Acidity (%) per 100g of fruit pulp of different *Annona* genotypes

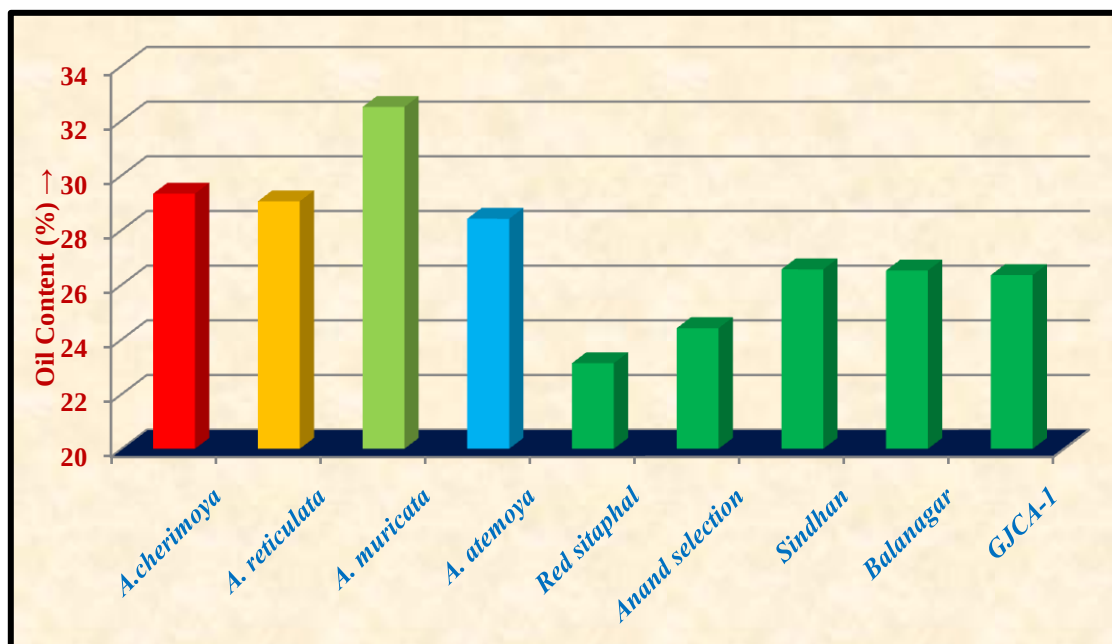


Fig 4.20 Oil content (%) per 100g of seeds of different *Annona* genotypes

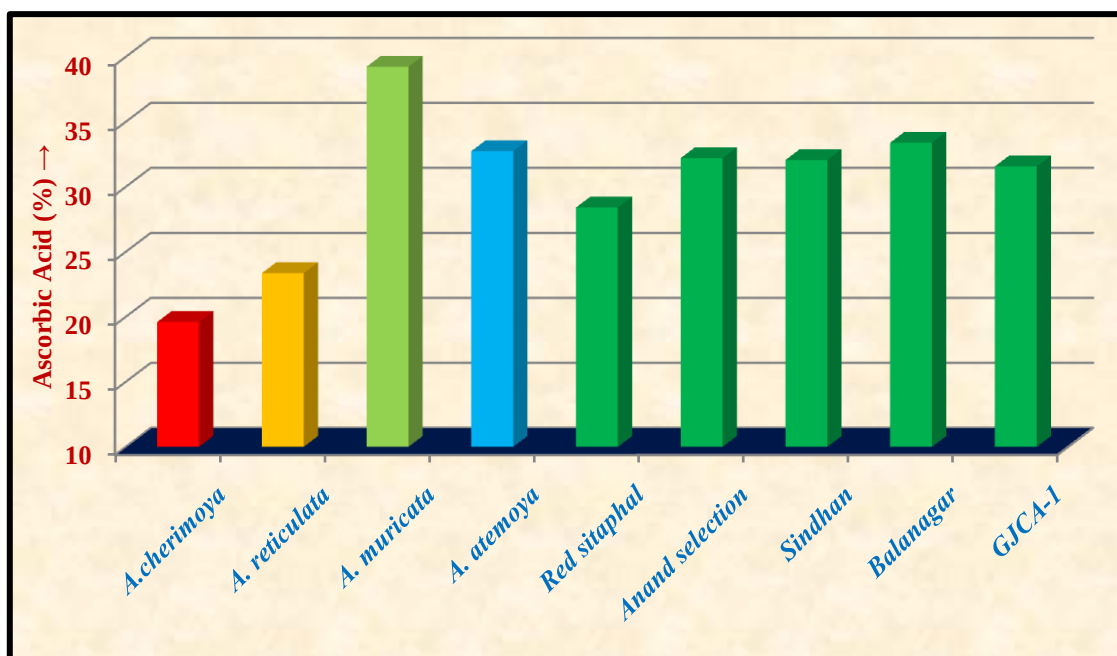


**Fig 4.21 Ascorbic acid content (%) per 100g of fruit pulp of different *Annona* genotypes**

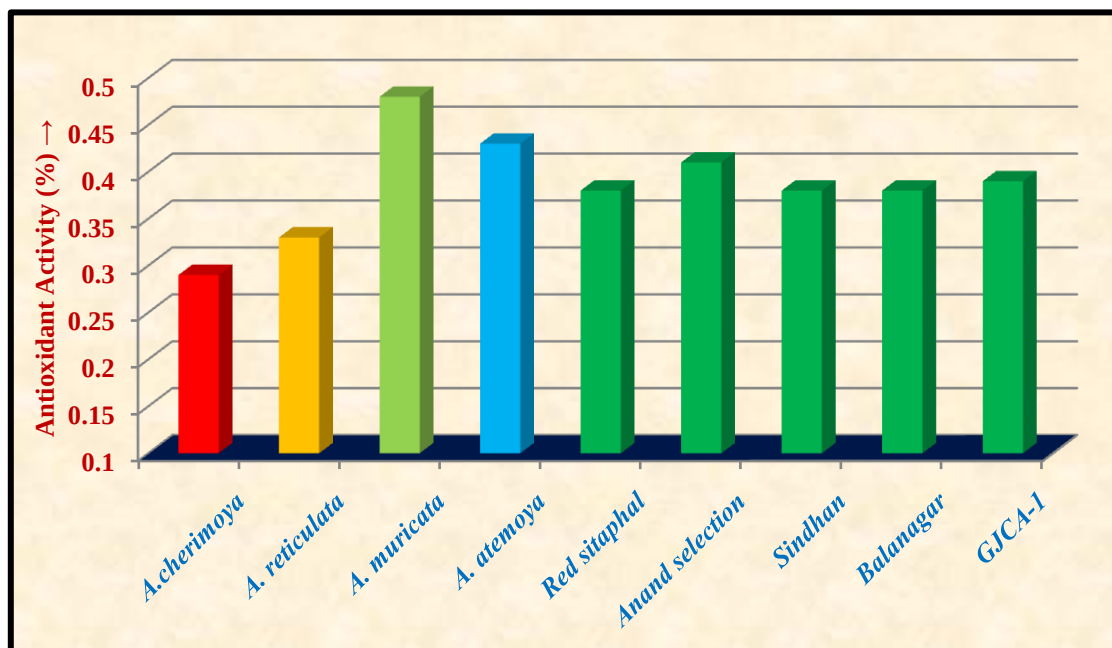


Fig 4.22 Antioxidant activity (%) per 100g of seeds of different *Annona* genotypes

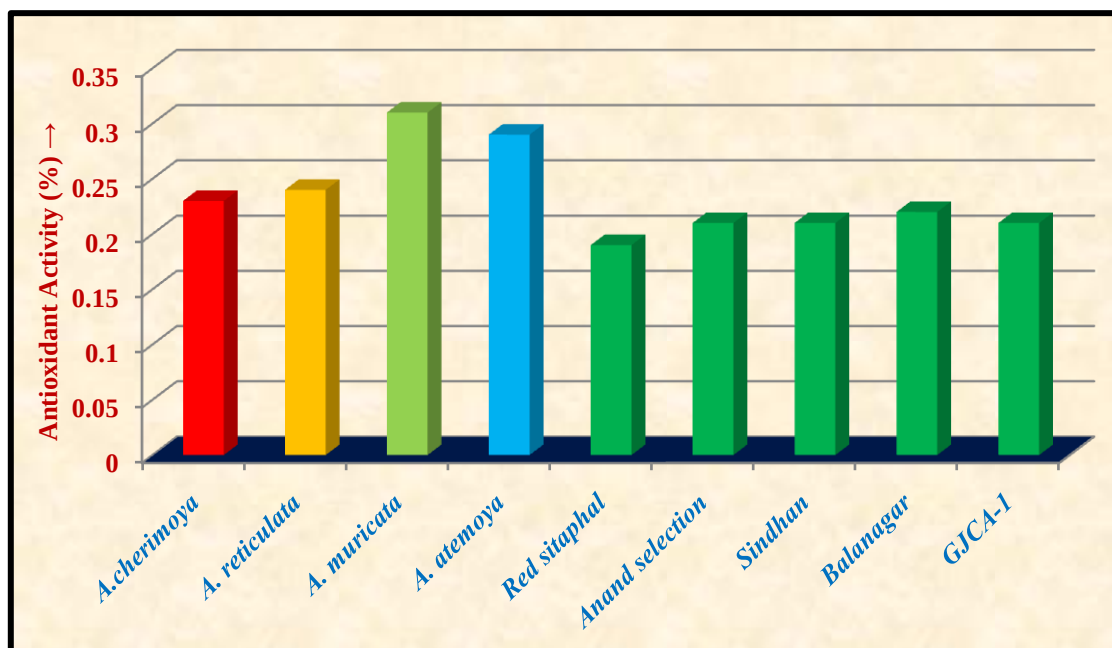


Fig 4.23 Antioxidant activity (%) per 100g of fruit pulp of different *Annona* genotypes

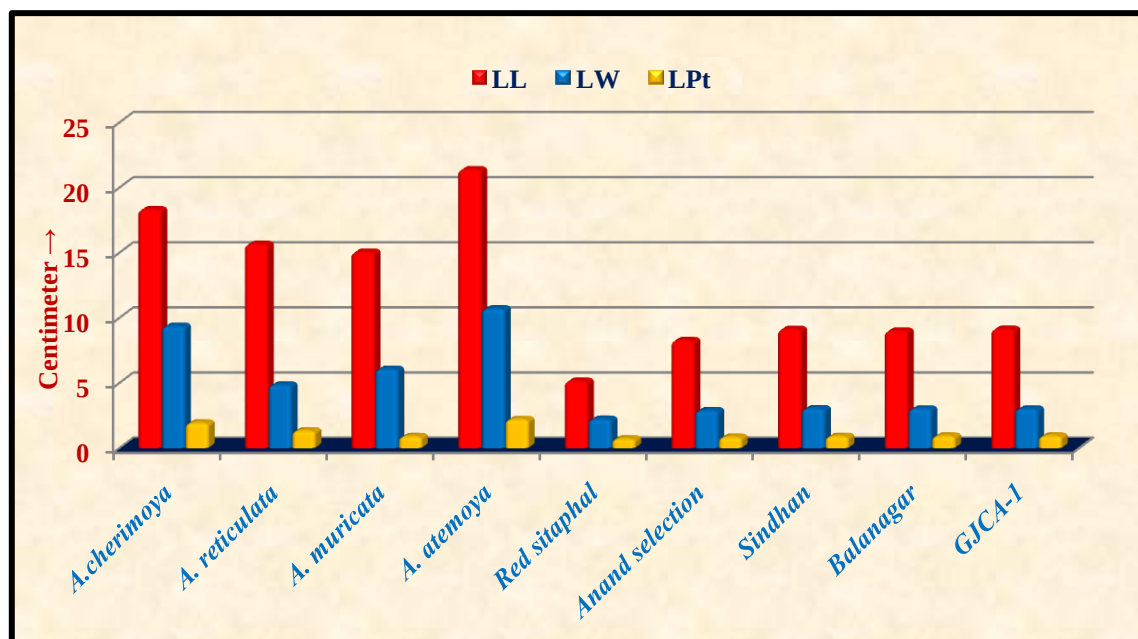


**Fig 4.2 Variation in Leaf length (LL), Leaf width (LW) and Length of petiole (LPt) for different *Annona* genotypes**

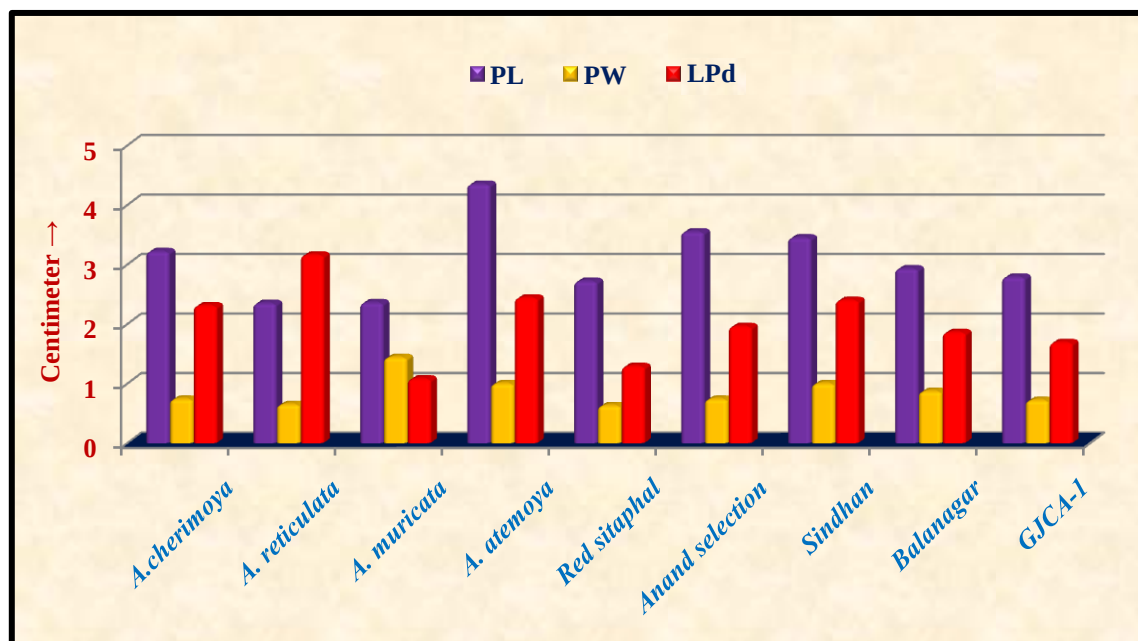


**Fig 4.3 Variation in Petal length (PL), Petal width (PW) and Length of pedicel (LPd) for different *Annona* genotypes**

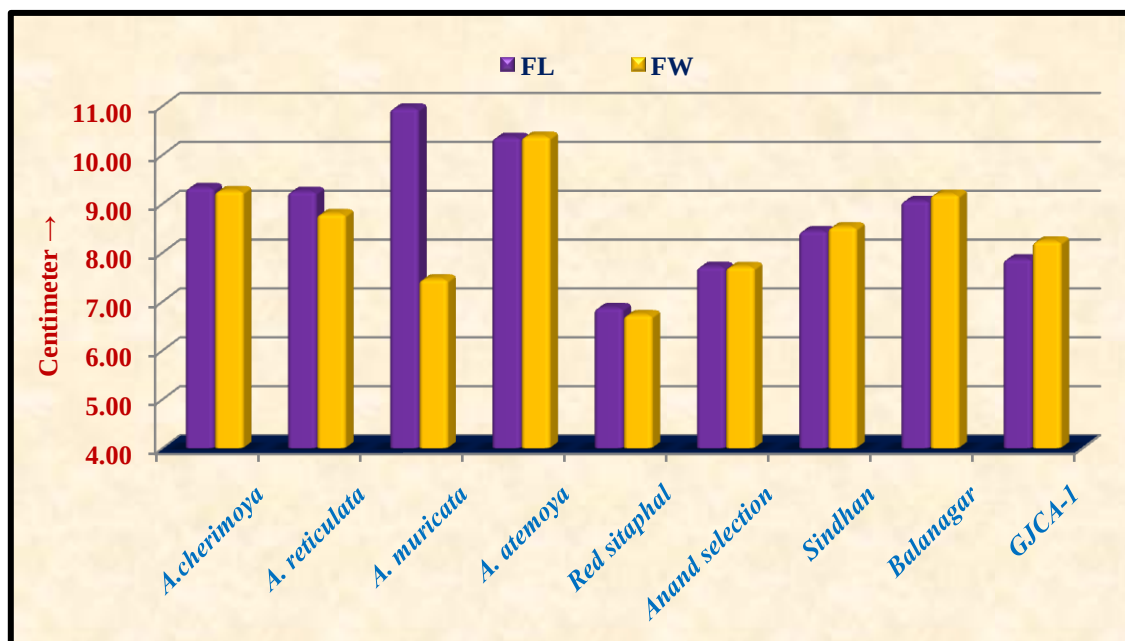


**Fig 4.6 Variation in Fruit length (FL) and Fruit width (FW) for different *Annona* genotypes**

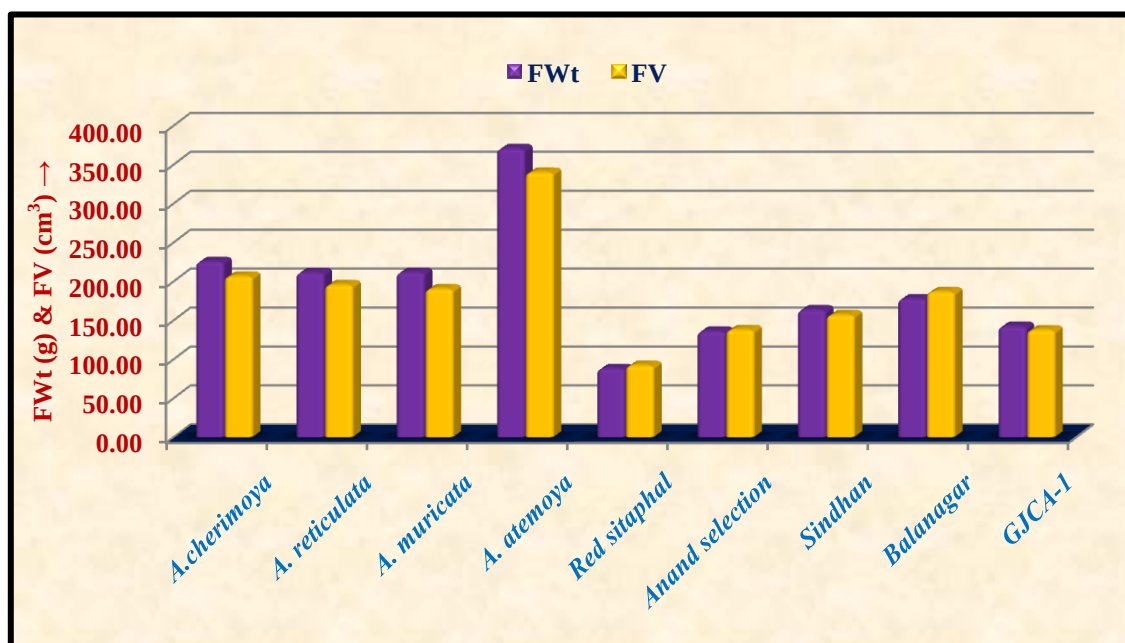


**Fig 4.7 Variation in Fruit weight (FWt) and Fruit volume (FV) for different *Annona* genotypes**

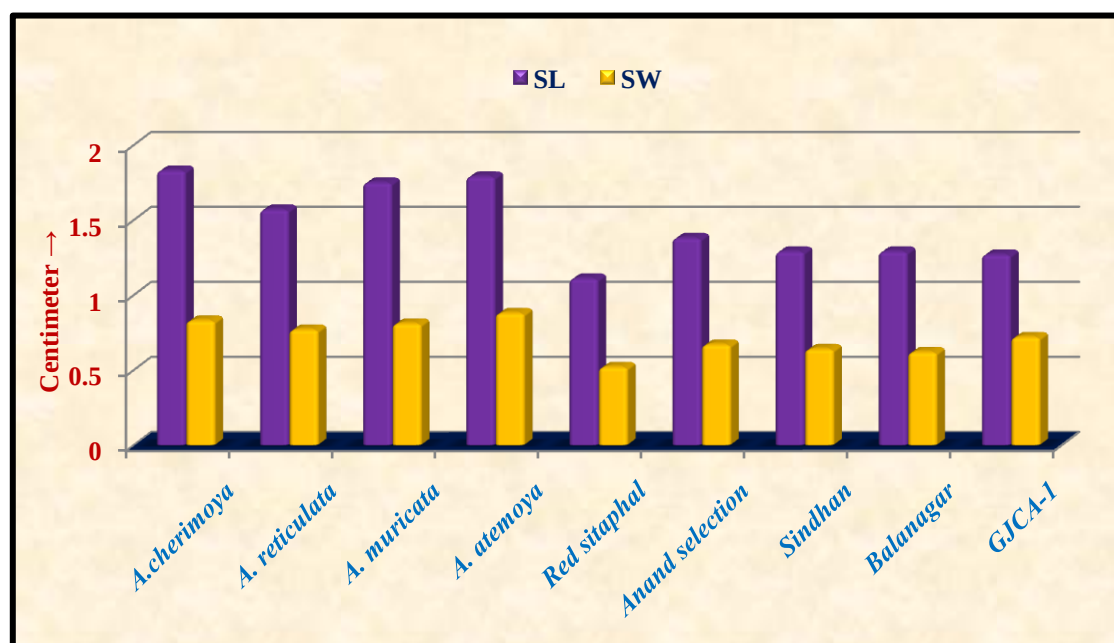


**Fig 4.9 Variation in Seed length (SL) and Seed width (SW) for different genotypes of *Annona* species**

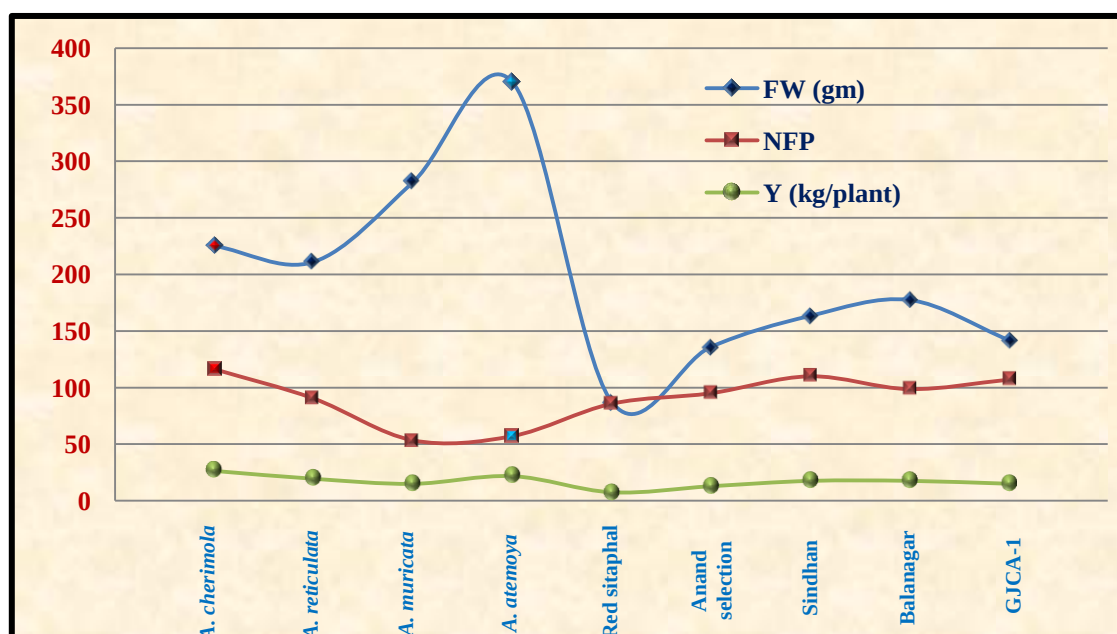
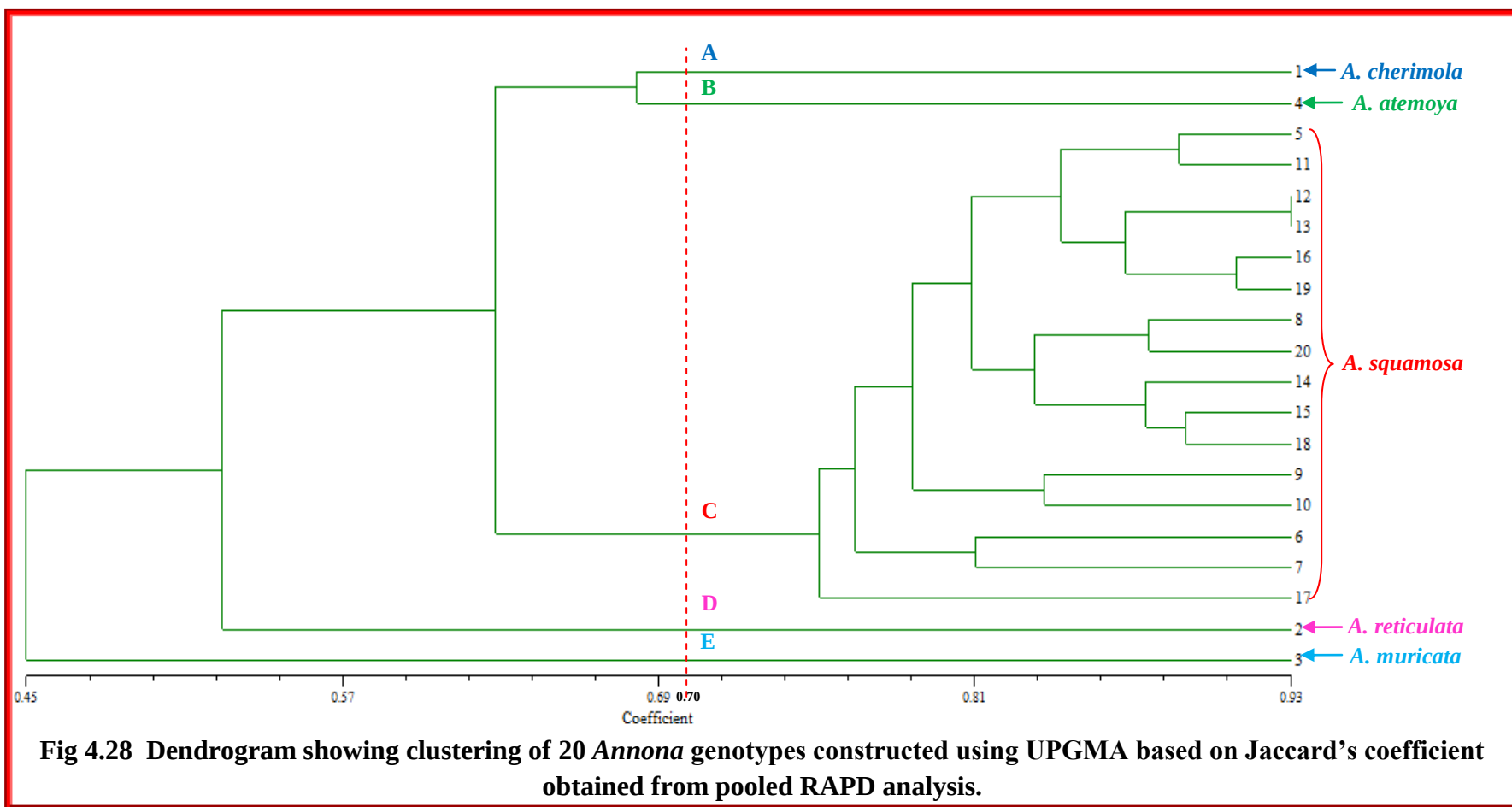


**Fig 4.10 Variation in Fruit weight (FW), No. of fruits per plant (NFP) and Fruit yield per plant (Y) for different *Annona* genotypes**

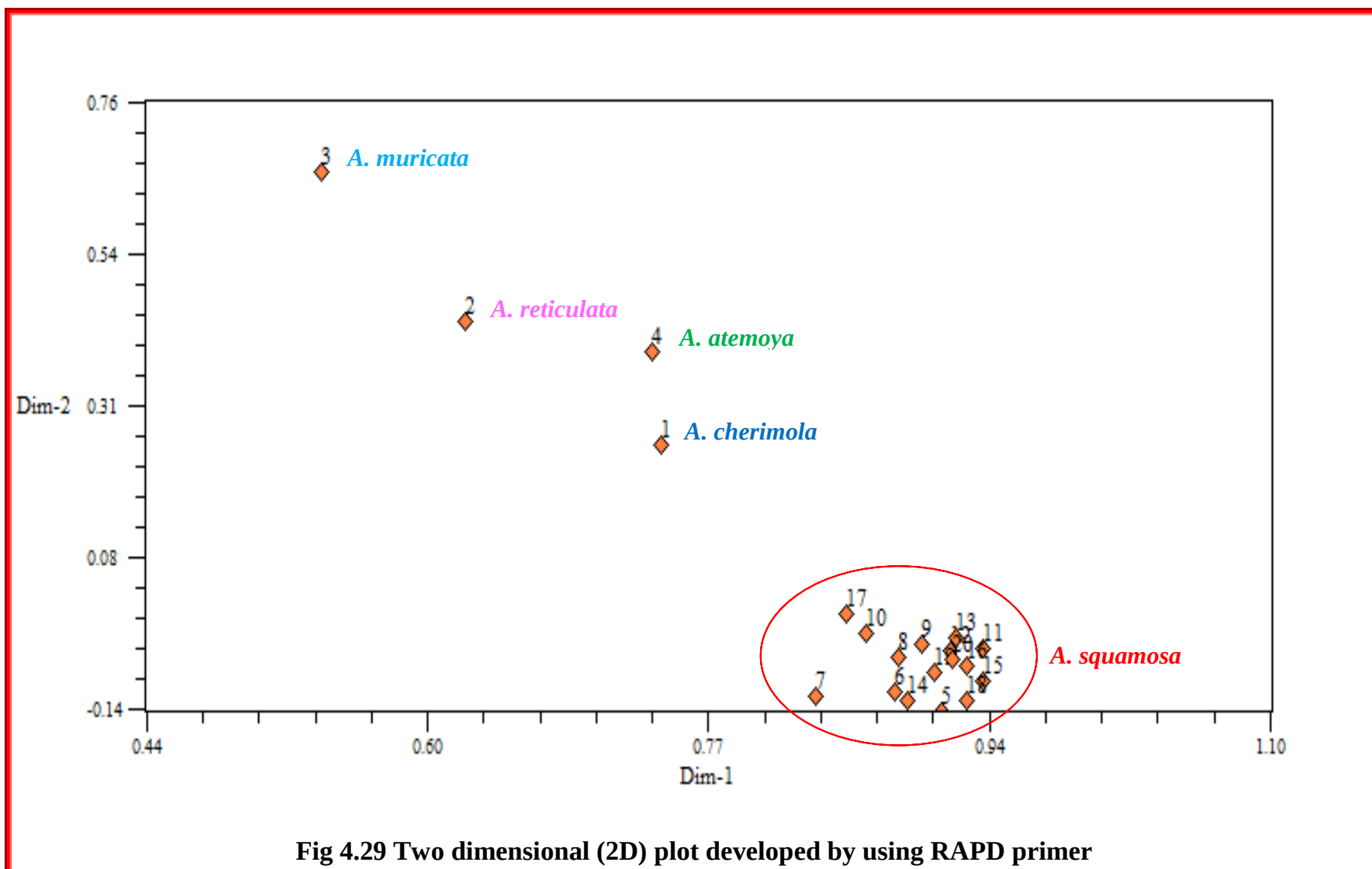


**Different species of *Annona*:**

1. *Annona cherimola* (A-1aa)
2. *Annona reticulata* (A-2)
3. *Annona muricata* (B)
4. *Annona atemoya* (A-1ab)
5. *Annona squamosa* (A-1b)

**Different varieties of *Annona squamosa*:**

- |                        |                      |           |                             |
|------------------------|----------------------|-----------|-----------------------------|
| 5. Red Sitaphal (R)    | 9. GJCA-1            | 13. ACC-3 | 17. Sindhan×Anand Selection |
| 6. Anand Selection (A) | 10. Vidyanagar local | 14. ACC-4 | 18. Sindhan×Balanagar       |
| 7. Sindhan (S)         | 11. ACC-1            | 15. ACC-5 | 19. Anand×Balanagar         |
| 8. Balanagar (B)       | 12. ACC-2            | 16. ACC-6 | 20. Balanagar×Red Sitaphal  |



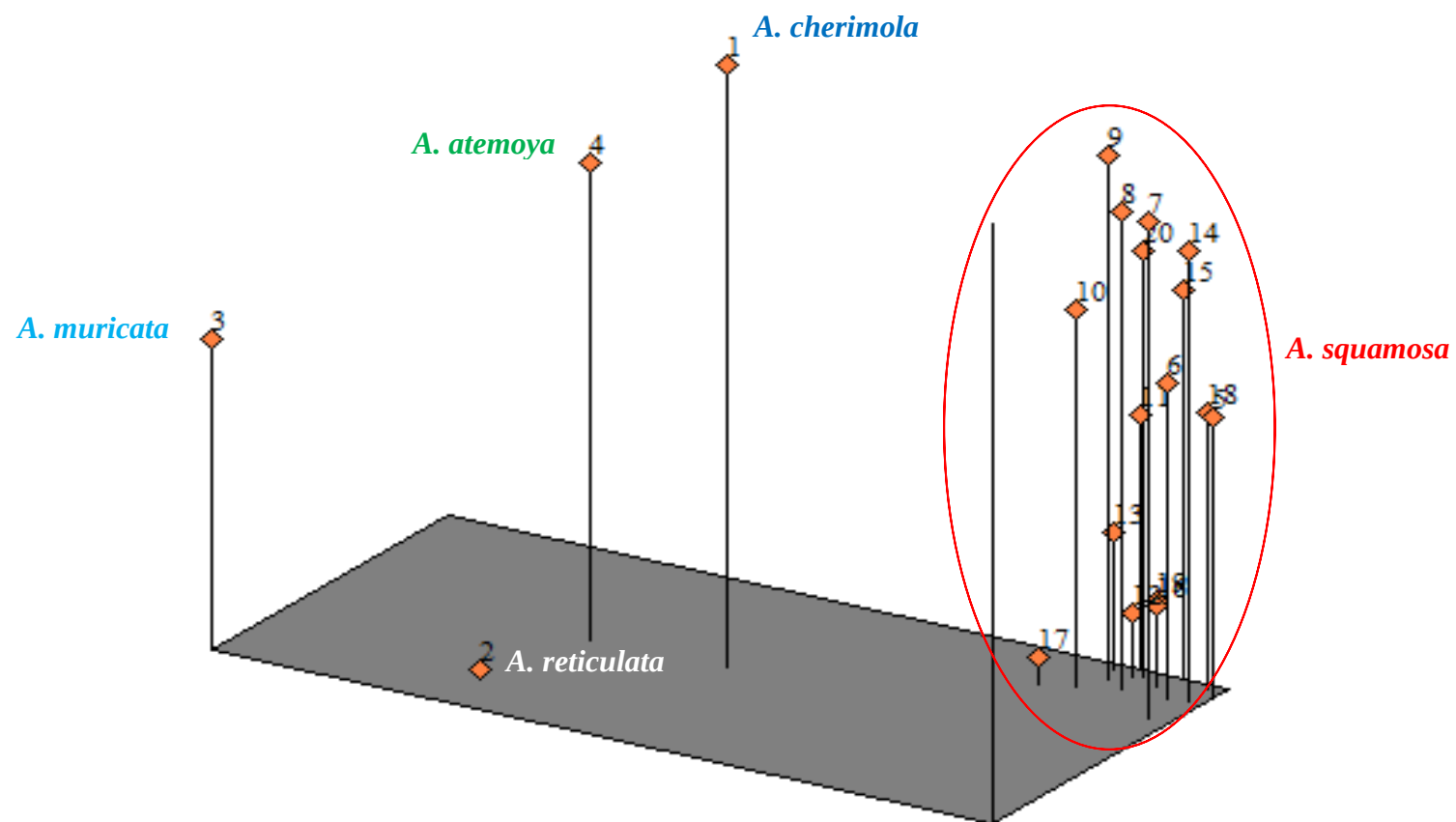


Fig 4.30 Three dimensional (3D) plot developed by using RAPD primers



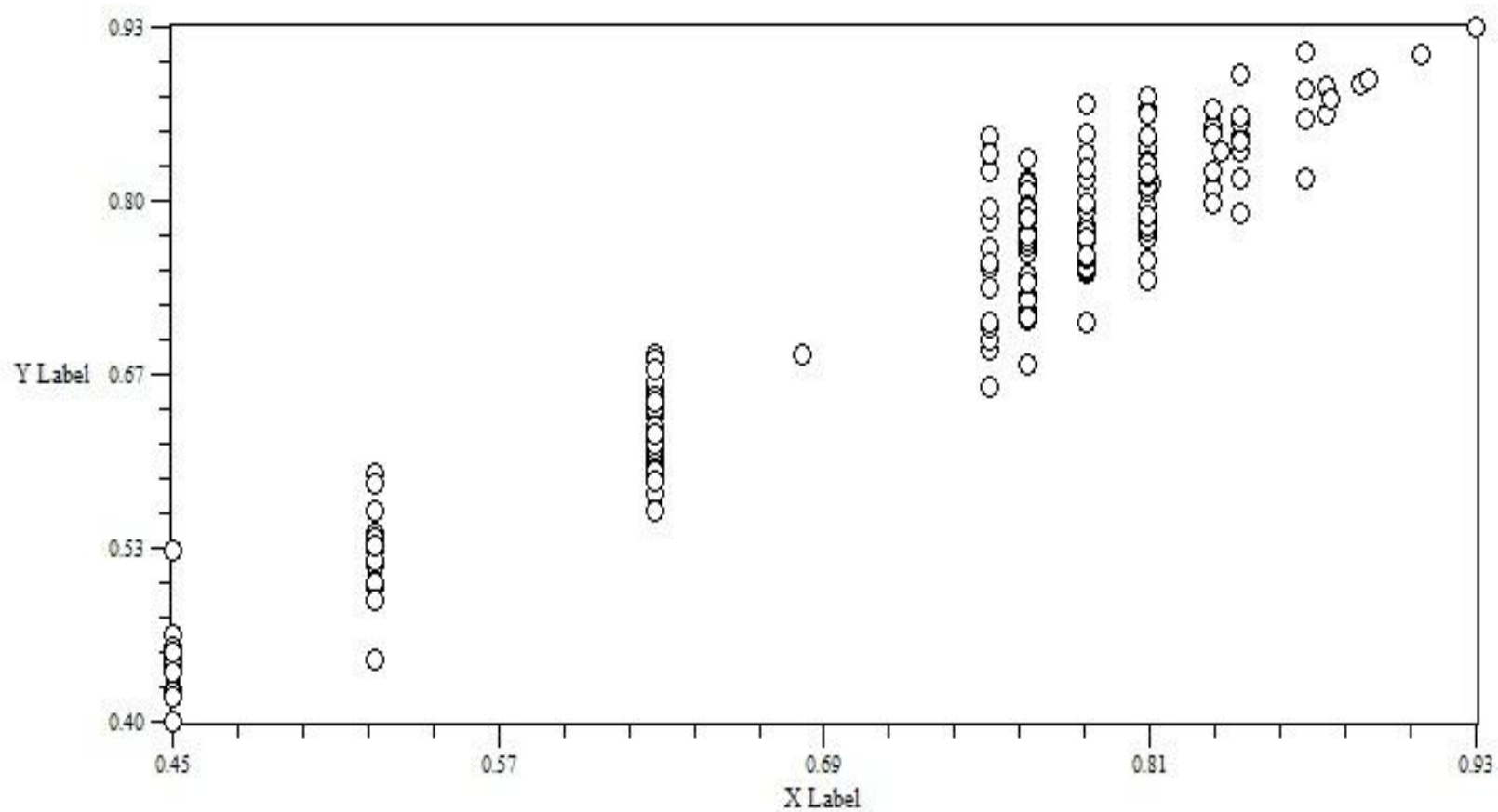
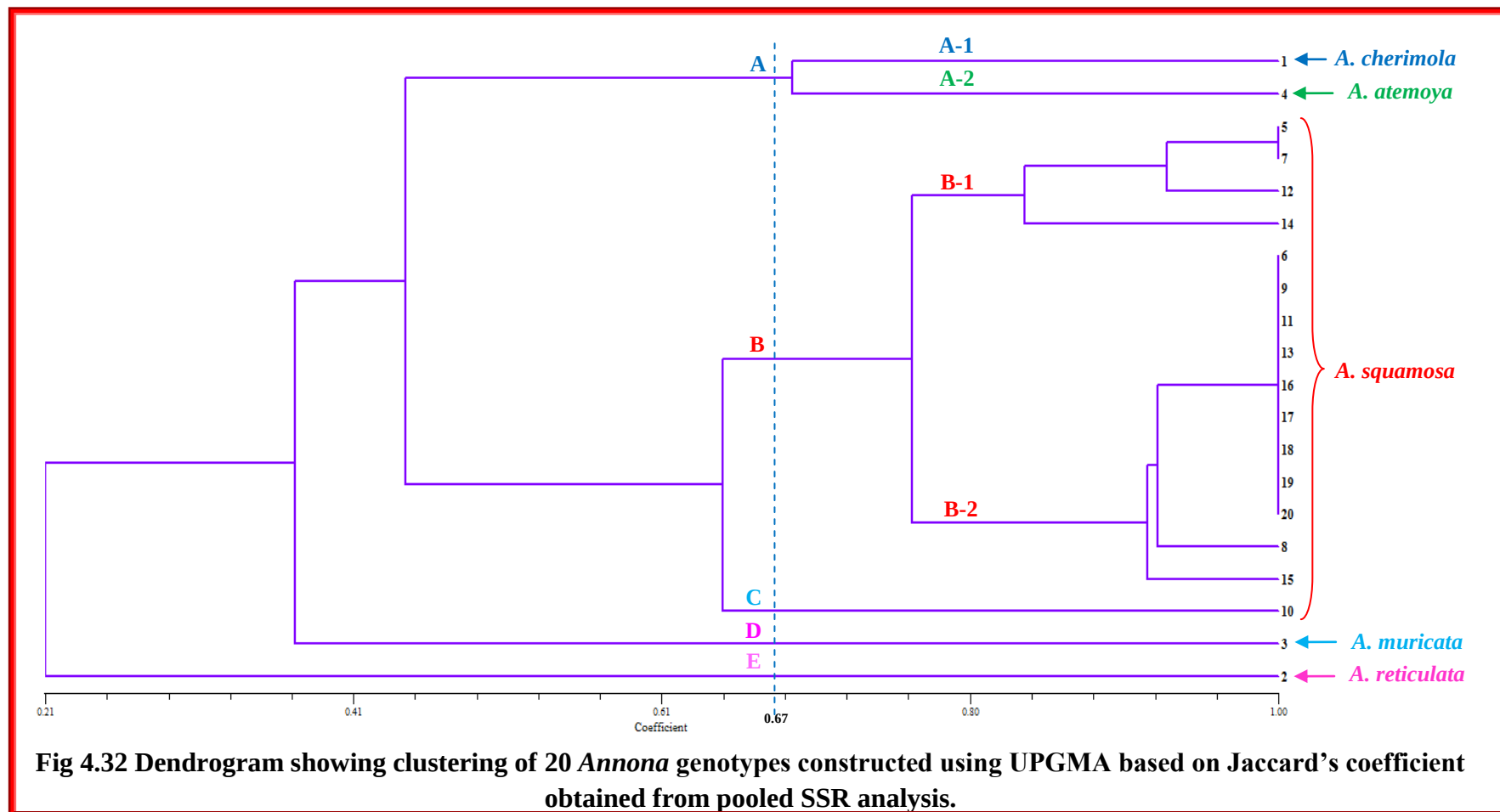


**Fig 4.31. Scatter-plot showing cophenetic correlation of RAPD markers**

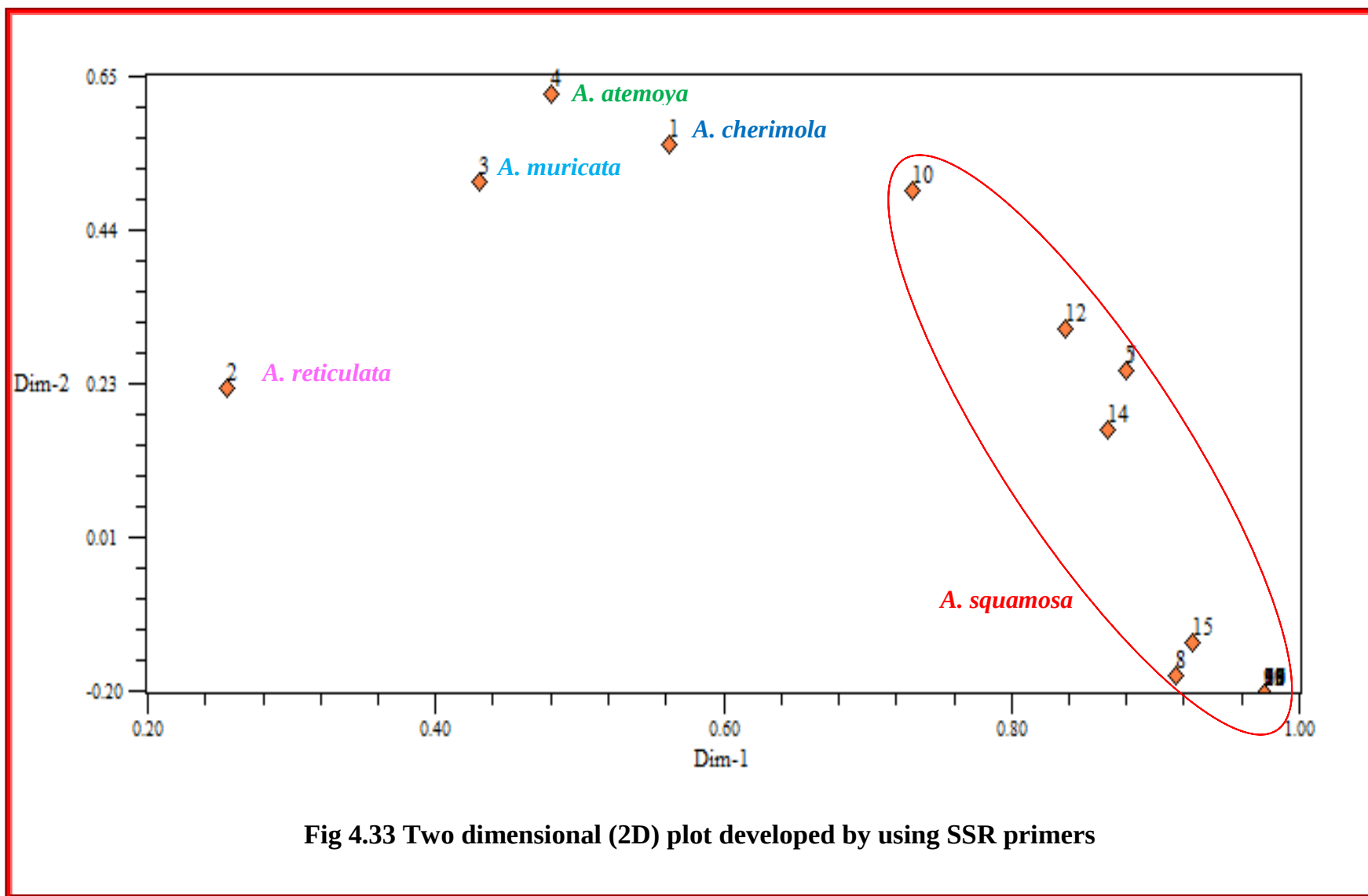


**Different species of *Annona*:**

1. *Annona cherimola* (A-1)
2. *Annona reticulata* (E)
3. *Annona muricata* (D)
4. *Annona atemoya* (A-2)
5. *Annona squamosa* (B & C)

**Different varieties of *Annona squamosa*:**

- |                        |                      |           |                               |
|------------------------|----------------------|-----------|-------------------------------|
| 5. Red Sitaphal (R)    | 9. GJCA-1            | 13. ACC-3 | 17. Sindhan×Anand Selection   |
| 6. Anand Selection (A) | 10. Vidyanagar local | 14. ACC-4 | 18. Sindhan×Balanagar         |
| 7. Sindhan (S)         | 11. ACC-1            | 15. ACC-5 | 19. Anand Selection×Balanagar |
| 8. Balanagar (B)       | 12. ACC-2            | 16. ACC-6 | 20. Balanagar×Red Sitaphal    |



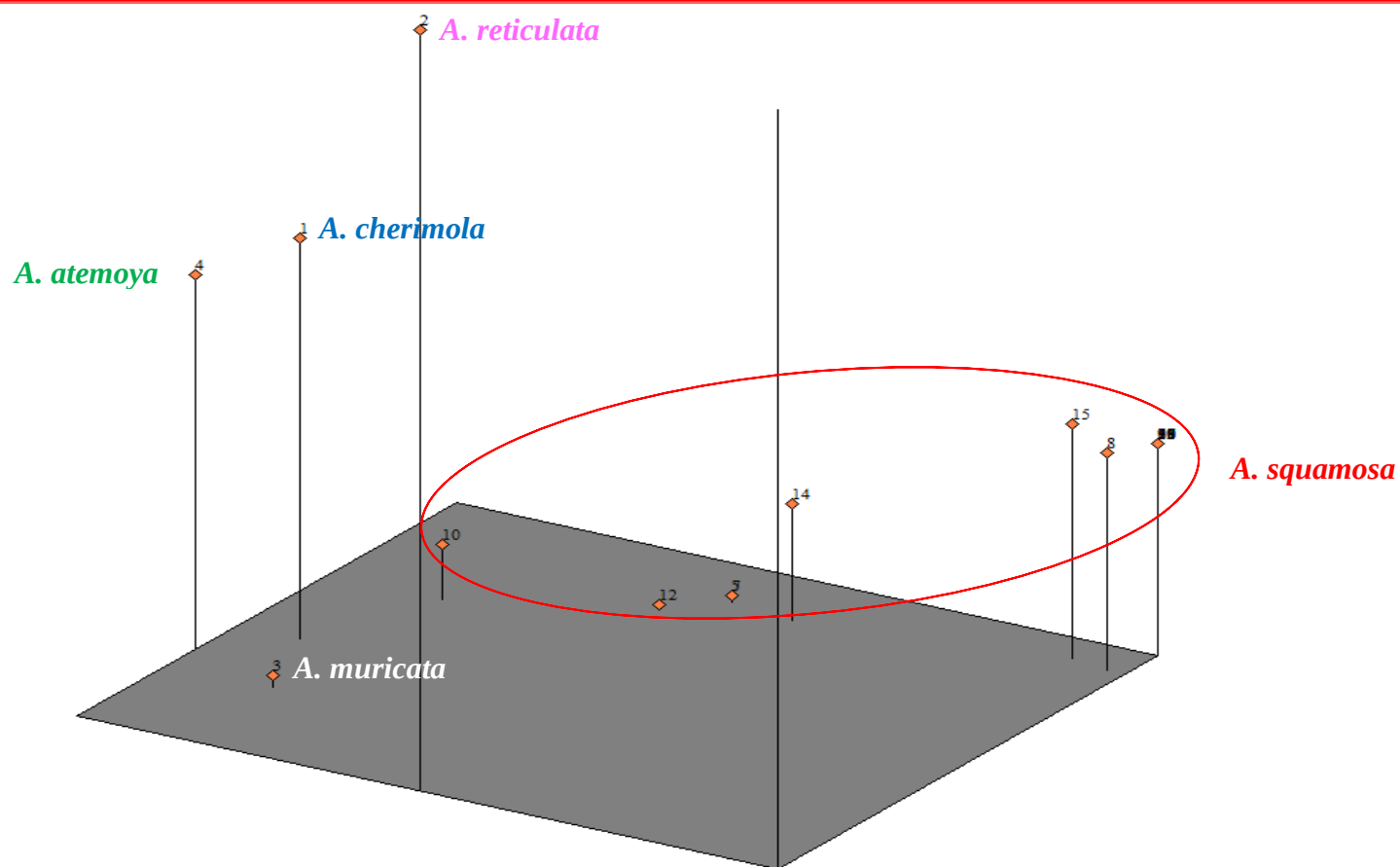


**Fig 4.34 Three dimensional (3D) plot developed by using SSR primers**

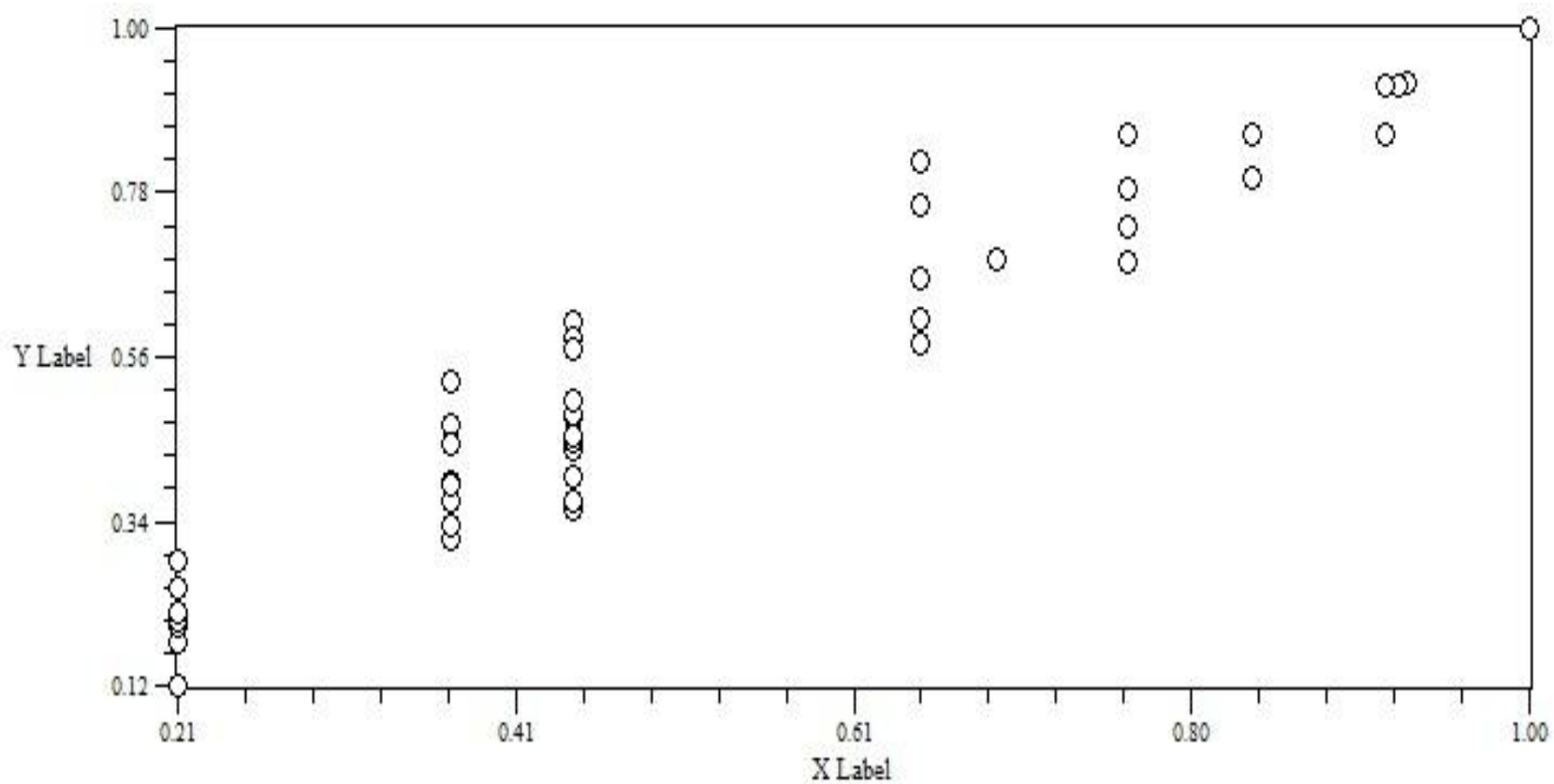


**Fig 4.35. Scatter-plot showing cophenetic correlation of SSR markers**

### DNA fingerprinting profiles of 20 *Annona* genotypes generated by RAPD primers

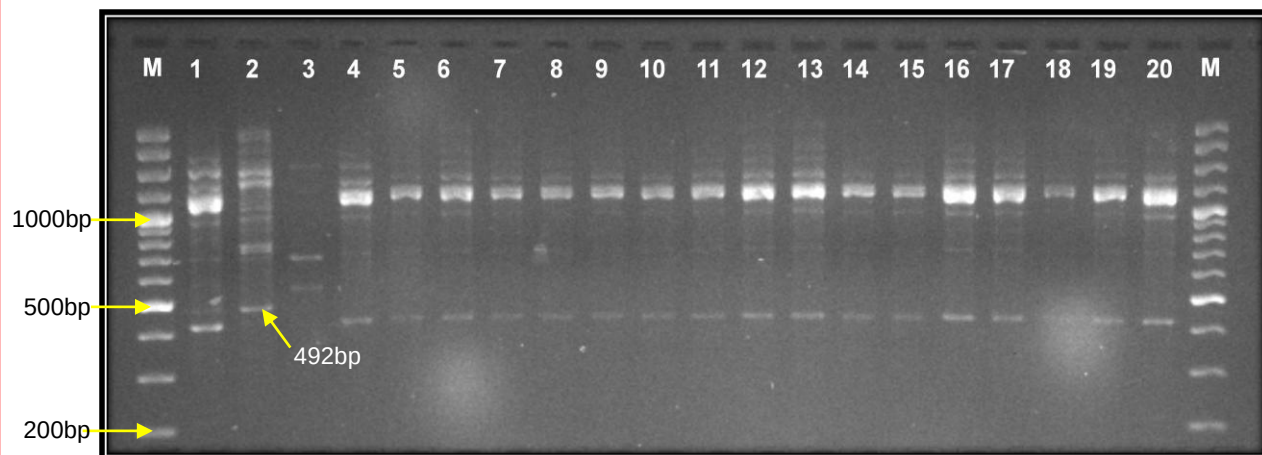


**Plate No-1. DNA fingerprinting profile generated by RAPD primer-OPB-6**

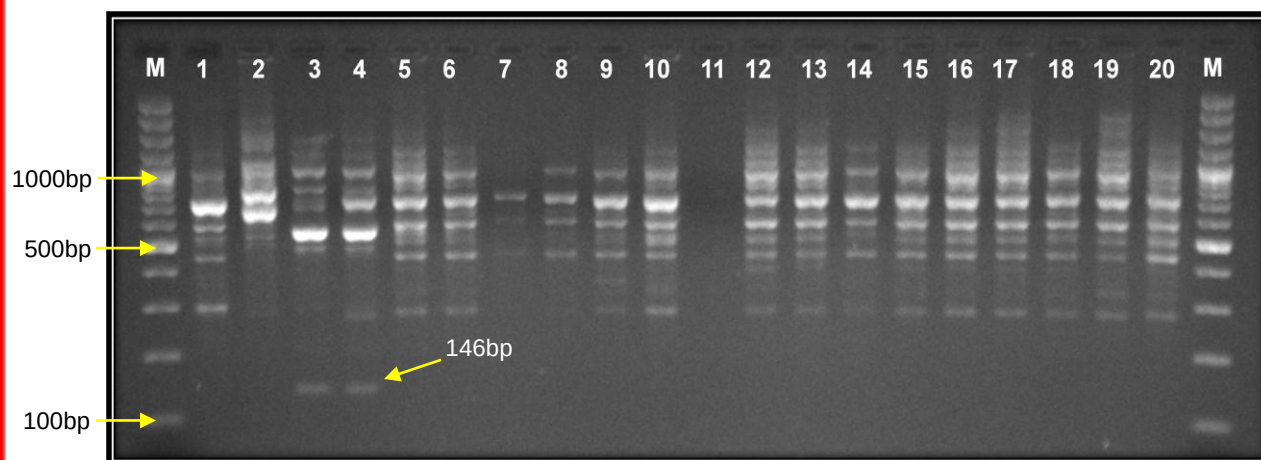


**Plate No-2. DNA fingerprinting profile generated by RAPD primer-OPB-7**

**M:-** 100+ bp ladder

#### List of 20 *Annona* genotypes

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 8.    | Balanagar        | 15.   | ACC-5                     |
| 2.    | <i>Annona reticulata</i> | 9.    | GJCA-1           | 16.   | ACC-6                     |
| 3.    | <i>Annona muricata</i>   | 10.   | Vidyanagar local | 17.   | Sindhan×Anand selection   |
| 4.    | <i>Annona atemoya</i>    | 11.   | ACC-1            | 18.   | Sindhan×Balanagar         |
| 5.    | Red sitaphal             | 12.   | ACC-2            | 19.   | Anand selection×Balanagar |
| 6.    | Anand selection          | 13.   | ACC-3            | 20.   | Balanagar×Red Sitaphal    |
| 7.    | Sindhan                  | 14.   | ACC-4            |       |                           |

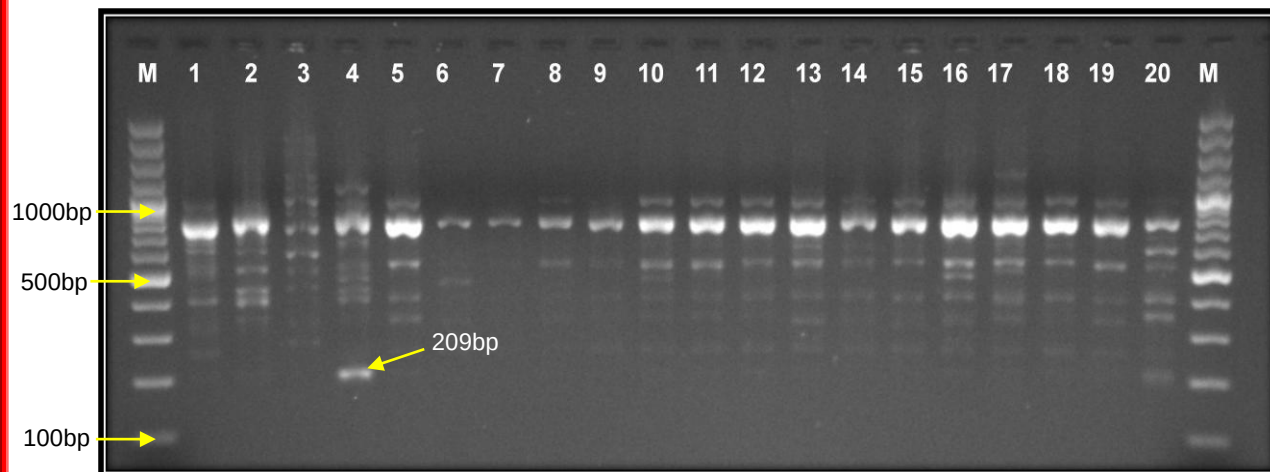


**Plate No-3. DNA fingerprinting profile generated by RAPD primer-OPB-8**

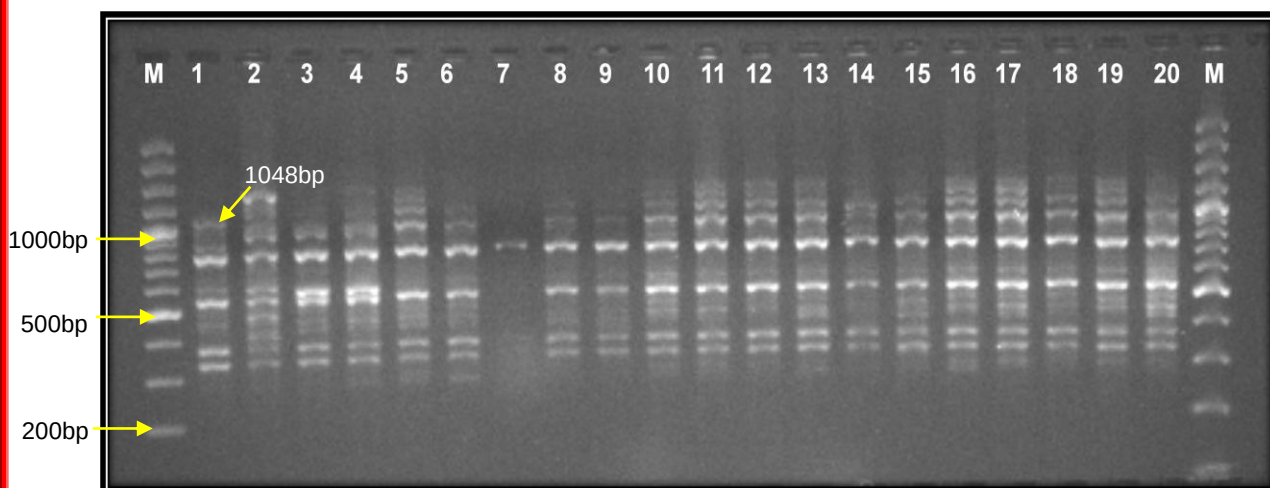


**Plate No-4. DNA fingerprinting profile generated by RAPD primer-OPB-10**

**M:-** 100+ bp ladder

**List of 20 *Annona* genotypes**

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 8.    | Balanagar        | 15.   | ACC-5                     |
| 2.    | <i>Annona reticulata</i> | 9.    | GJCA-1           | 16.   | ACC-6                     |
| 3.    | <i>Annona muricata</i>   | 10.   | Vidyanagar local | 17.   | Sindhani×Anand selection  |
| 4.    | <i>Annona atemoya</i>    | 11.   | ACC-1            | 18.   | Sindhani×Balanagar        |
| 5.    | Red sitaphal             | 12.   | ACC-2            | 19.   | Anand selection×Balanagar |
| 6.    | Anand selection          | 13.   | ACC-3            | 20.   | Balanagar×Red Sitaphal    |
| 7.    | Sindhani                 | 14.   | ACC-4            |       |                           |

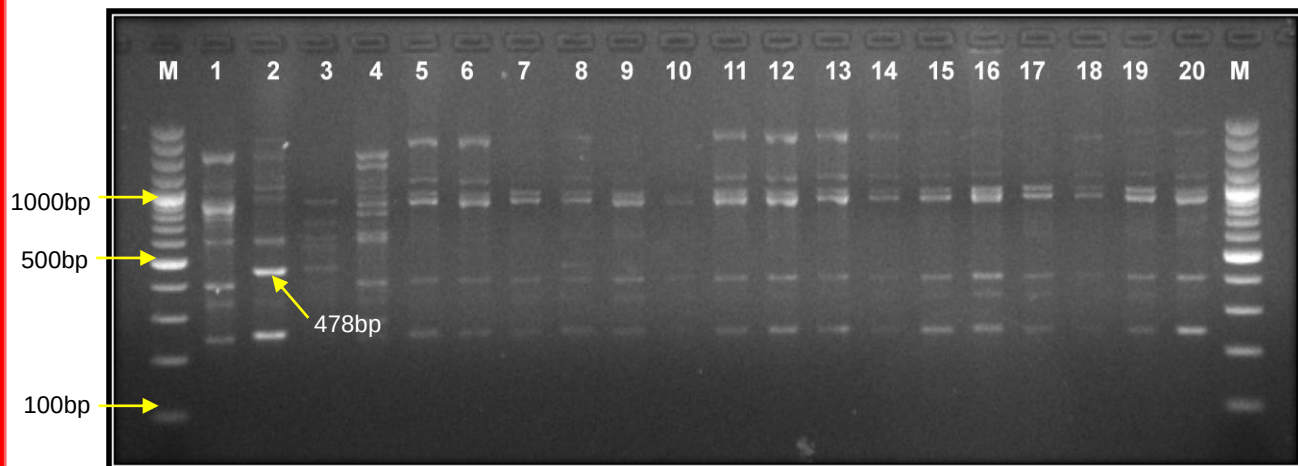


**Plate No-5. DNA fingerprinting profile generated by RAPD primer-OPB-12**

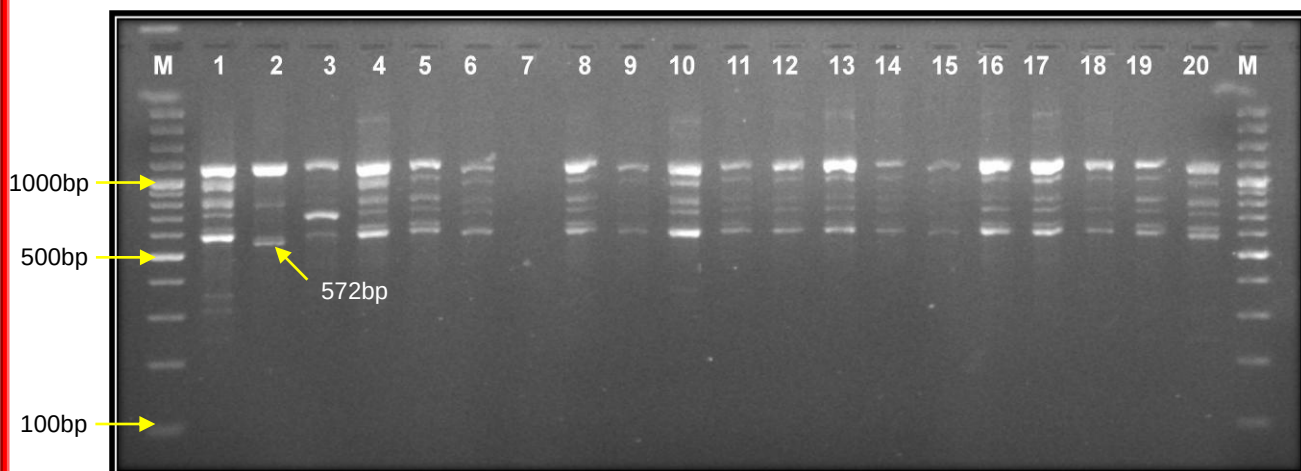


**Plate No-6. DNA fingerprinting profile generated by RAPD primer-OPD-2**

**M:-** 100+ bp ladder

**List of 20 *Annona* genotypes**

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 8.    | Balanagar        | 15.   | ACC-5                     |
| 2.    | <i>Annona reticulata</i> | 9.    | GJCA-1           | 16.   | ACC-6                     |
| 3.    | <i>Annona muricata</i>   | 10.   | Vidyanagar local | 17.   | Sindhan×Anand selection   |
| 4.    | <i>Annona atemoya</i>    | 11.   | ACC-1            | 18.   | Sindhan×Balanagar         |
| 5.    | Red sitaphal             | 12.   | ACC-2            | 19.   | Anand selection×Balanagar |
| 6.    | Anand selection          | 13.   | ACC-3            | 20.   | Balanagar×Red sitaphal    |
| 7.    | Sindhan                  | 14.   | ACC-4            |       |                           |

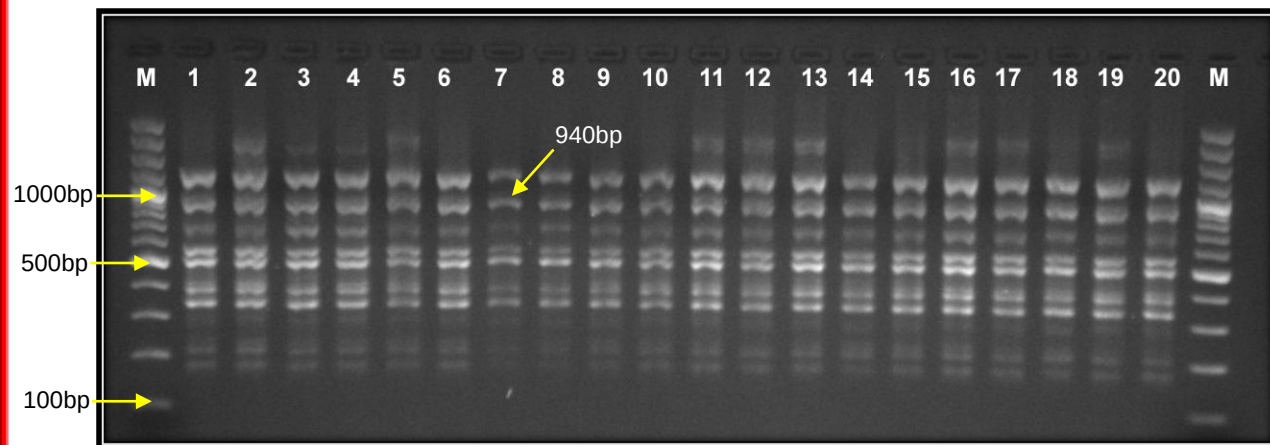


**Plate No-7. DNA fingerprinting profile generated by RAPD primer-OPD-16**

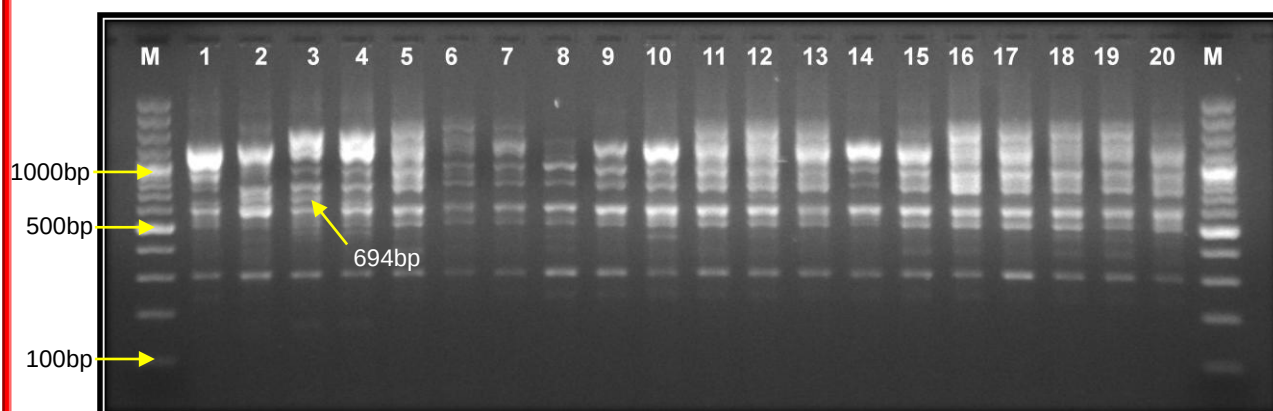


**Plate No-8. DNA fingerprinting profile generated by RAPD primer-OPD-20**

**M:-** 100+ bp ladder

**List of 20 *Annona* genotypes**

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 8.    | Balanagar        | 15.   | ACC-5                     |
| 2.    | <i>Annona reticulata</i> | 9.    | GJCA-1           | 16.   | ACC-6                     |
| 3.    | <i>Annona muricata</i>   | 10.   | Vidyanagar local | 17.   | Sindhan×Anand selection   |
| 4.    | <i>Annona atemoya</i>    | 11.   | ACC-1            | 18.   | Sindhan×Balanagar         |
| 5.    | Red sitaphal             | 12.   | ACC-2            | 19.   | Anand selection×Balanagar |
| 6.    | Anand selection          | 13.   | ACC-3            | 20.   | Balanagar×Red sitaphal    |
| 7.    | Sindhan                  | 14.   | ACC-4            |       |                           |

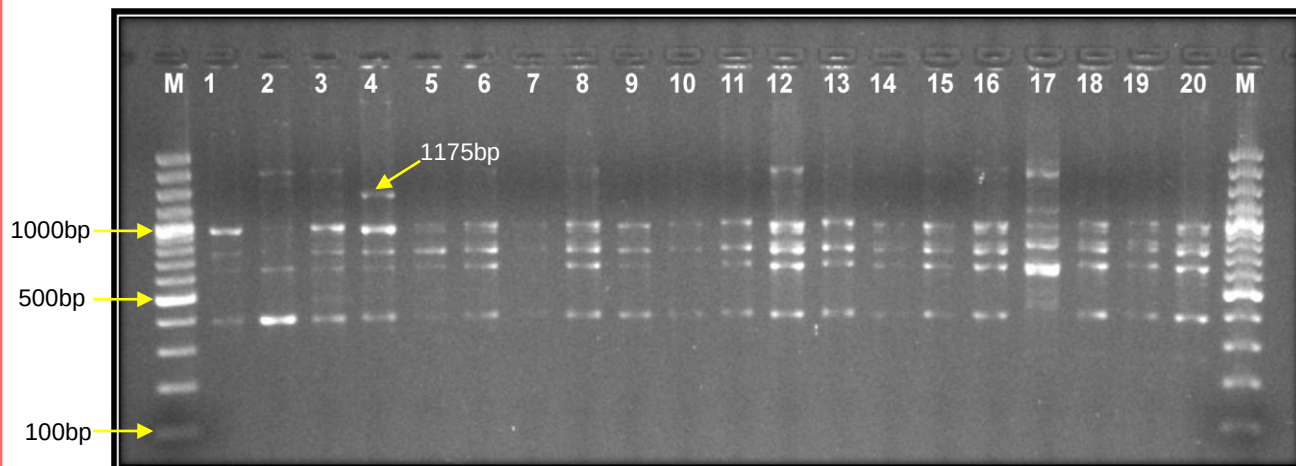


**Plate No-9. DNA fingerprinting profile generated by RAPD primer-OPF-4**

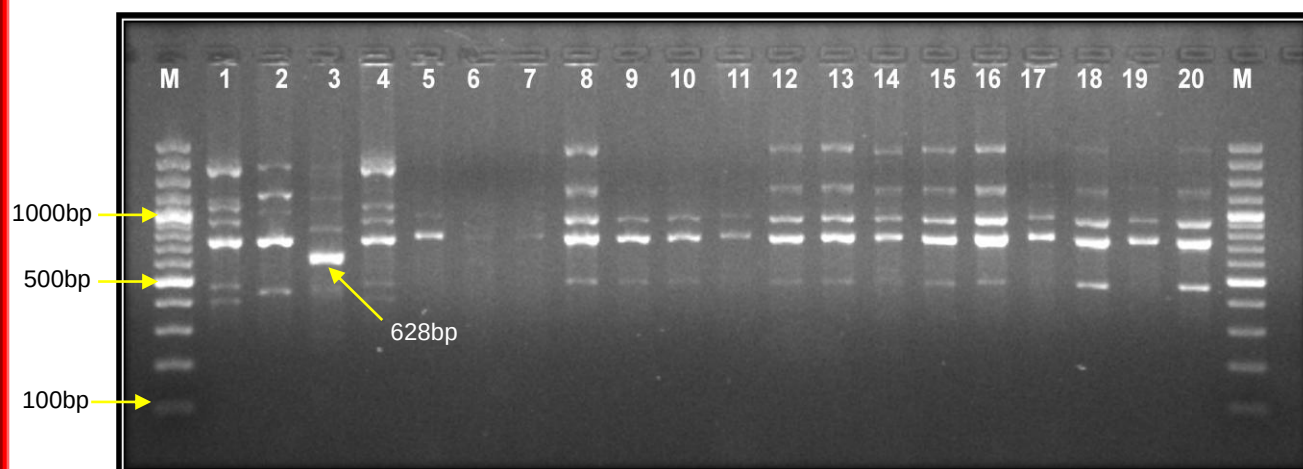
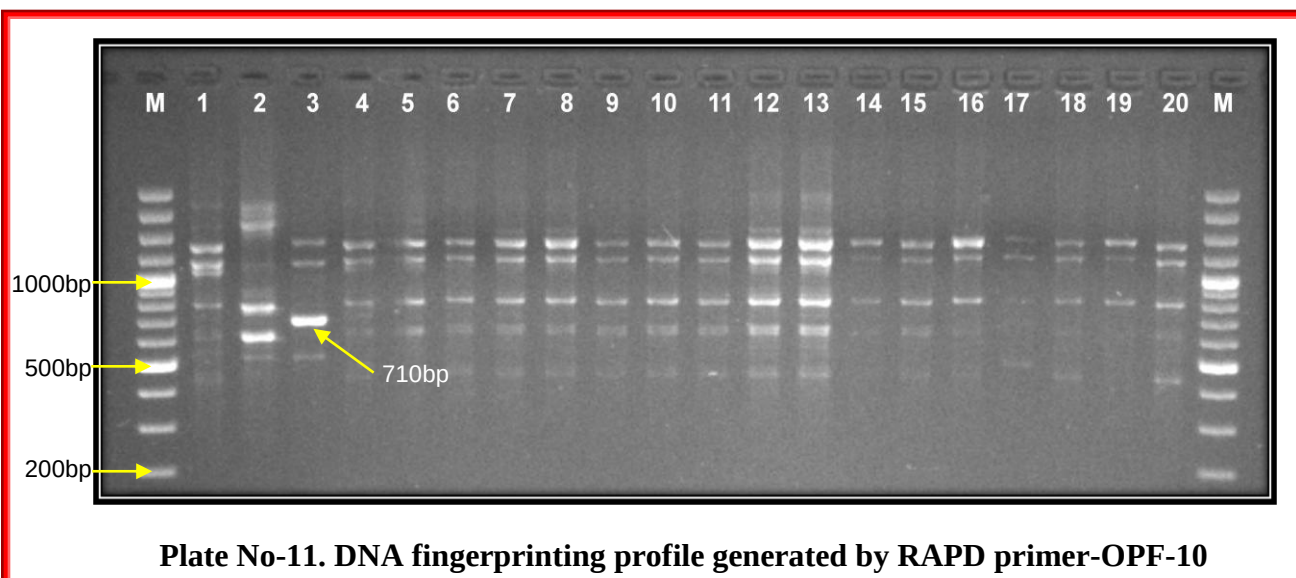


**Plate No-10. DNA fingerprinting profile generated by RAPD primer-OPF-5**

**M:-** 100+ bp ladder

**List of 20 *Annona* genotypes**

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 8.    | Balanagar        | 15.   | ACC-5                     |
| 2.    | <i>Annona reticulata</i> | 9.    | GJCA-1           | 16.   | ACC-6                     |
| 3.    | <i>Annona muricata</i>   | 10.   | Vidyanagar local | 17.   | Sindhan×Anand selection   |
| 4.    | <i>Annona atemoya</i>    | 11.   | ACC-1            | 18.   | Sindhan×Balanagar         |
| 5.    | Red sitaphal             | 12.   | ACC-2            | 19.   | Anand selection×Balanagar |
| 6.    | Anand selection          | 13.   | ACC-3            | 20.   | Balanagar×Red Sitaphal    |
| 7.    | Sindhan                  | 14.   | ACC-4            |       |                           |



**M:-** 100+ bp ladder

**List of 20 *Annona* genotypes**

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 8.    | Balanagar        | 15.   | ACC-5                     |
| 2.    | <i>Annona reticulata</i> | 9.    | GJCA-1           | 16.   | ACC-6                     |
| 3.    | <i>Annona muricata</i>   | 10.   | Vidyanagar local | 17.   | Sindhan×Anand selection   |
| 4.    | <i>Annona atemoya</i>    | 11.   | ACC-1            | 18.   | Sindhan×Balanagar         |
| 5.    | Red sitaphal             | 12.   | ACC-2            | 19.   | Anand selection×Balanagar |
| 6.    | Anand selection          | 13.   | ACC-3            | 20.   | Balanagar×Red Sitaphal    |
| 7.    | Sindhan                  | 14.   | ACC-4            |       |                           |

### DNA Fingerprinting profiles of 20 *Annona* genotypes generated by SSR primers

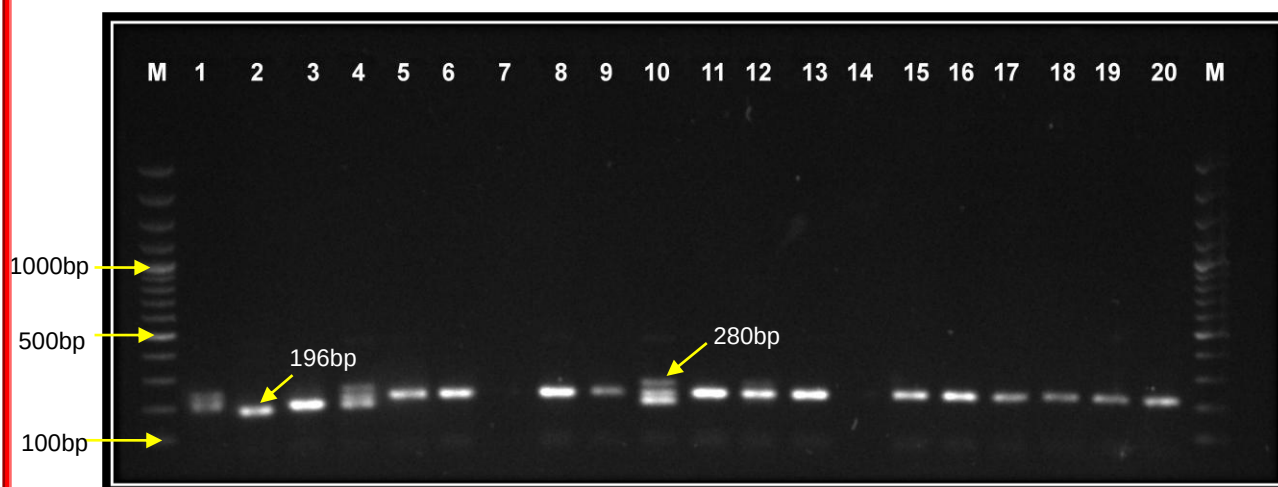


**Plate No-12. DNA fingerprinting profile generated by SSR primer-LMCH-29**

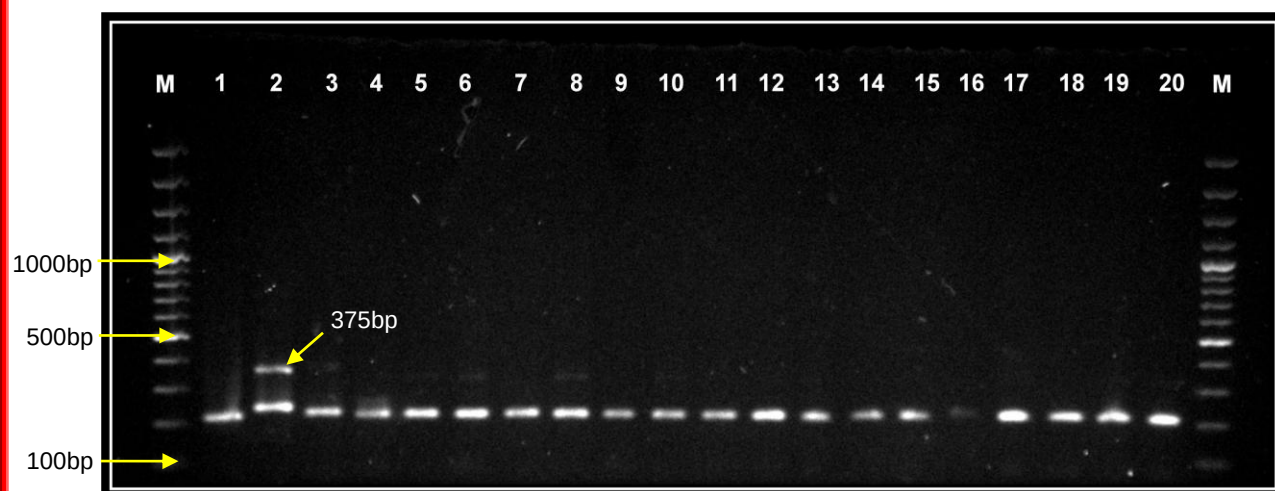


**Plate No-13. DNA fingerprinting profile generated by SSR primer-LMCH-43**

**M:-** 100+ bp ladder

#### List of 20 *Annona* genotypes

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 8.    | Balanagar        | 15.   | ACC-5                     |
| 2.    | <i>Annona reticulata</i> | 9.    | GJCA-1           | 16.   | ACC-6                     |
| 3.    | <i>Annona muricata</i>   | 10.   | Vidyanagar local | 17.   | Sindhan×Anand selection   |
| 4.    | <i>Annona atemoya</i>    | 11.   | ACC-1            | 18.   | Sindhan×Balanagar         |
| 5.    | Red sitaphal             | 12.   | ACC-2            | 19.   | Anand selection×Balanagar |
| 6.    | Anand selection          | 13.   | ACC-3            | 20.   | Balanagar×Red sitaphal    |
| 7.    | Sindhan                  | 14.   | ACC-4            |       |                           |

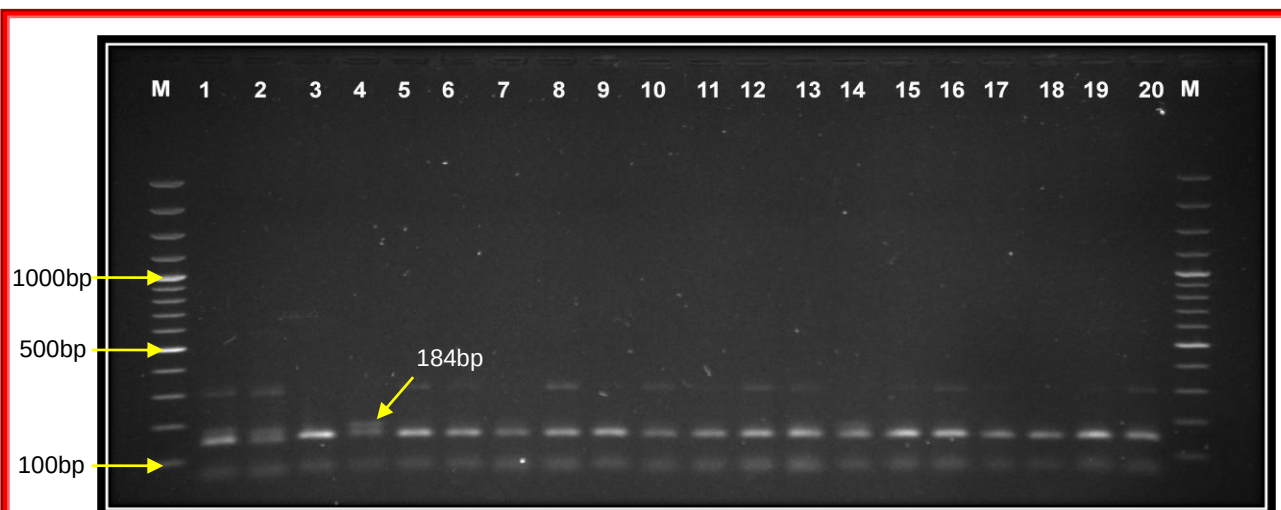


**Plate No-14. DNA fingerprinting profile generated by SSR primer-LMCH-48**

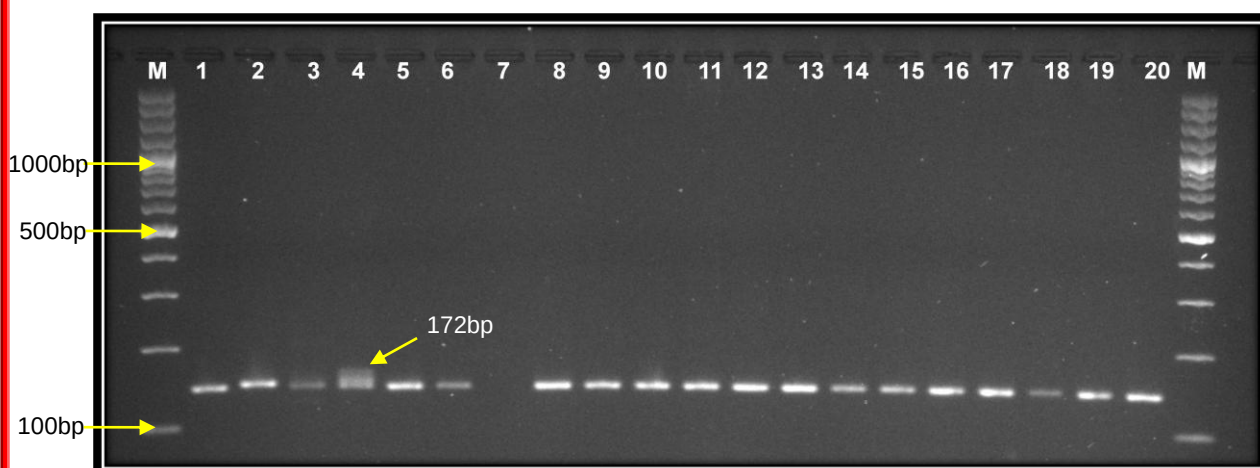


**Plate No-15. DNA fingerprinting profile generated by SSR primer-LMCH-70**

**M:-** 100+ bp ladder

**List of 20 *Annona* genotypes**

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 8.    | Balanagar        | 15.   | ACC-5                     |
| 2.    | <i>Annona reticulata</i> | 9.    | GJCA-1           | 16.   | ACC-6                     |
| 3.    | <i>Annona muricata</i>   | 10.   | Vidyanagar local | 17.   | Sindhan×Anand selection   |
| 4.    | <i>Annona atemoya</i>    | 11.   | ACC-1            | 18.   | Sindhan×Balanagar         |
| 5.    | Red sitaphal             | 12.   | ACC-2            | 19.   | Anand selection×Balanagar |
| 6.    | Anand selection          | 13.   | ACC-3            | 20.   | Balanagar×Red sitaphal    |
| 7.    | Sindhan                  | 14.   | ACC-4            |       |                           |

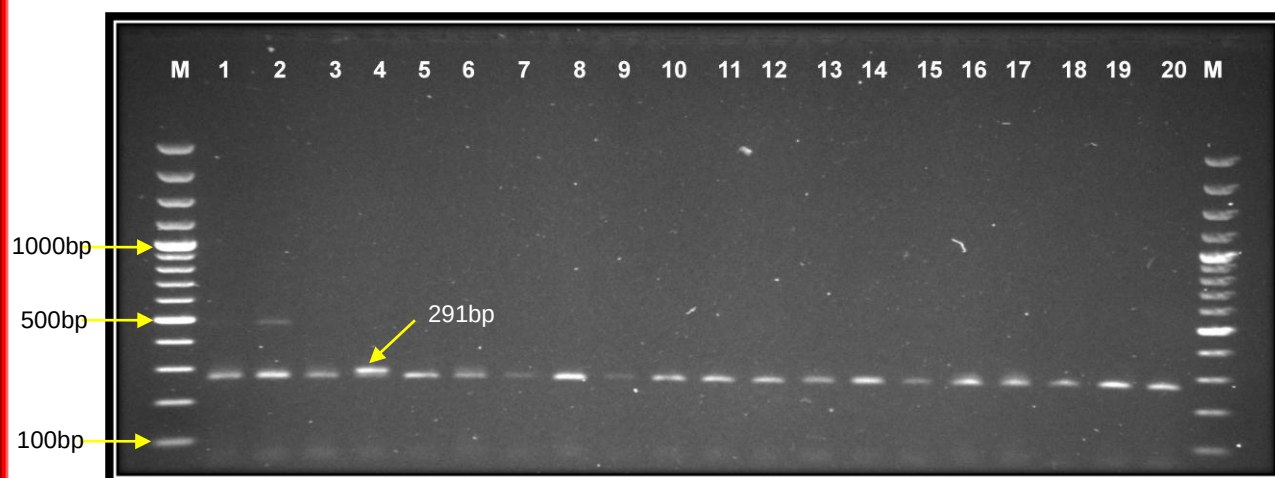


**Plate No-16. DNA fingerprinting profile generated by SSR primer-LMCH-71**

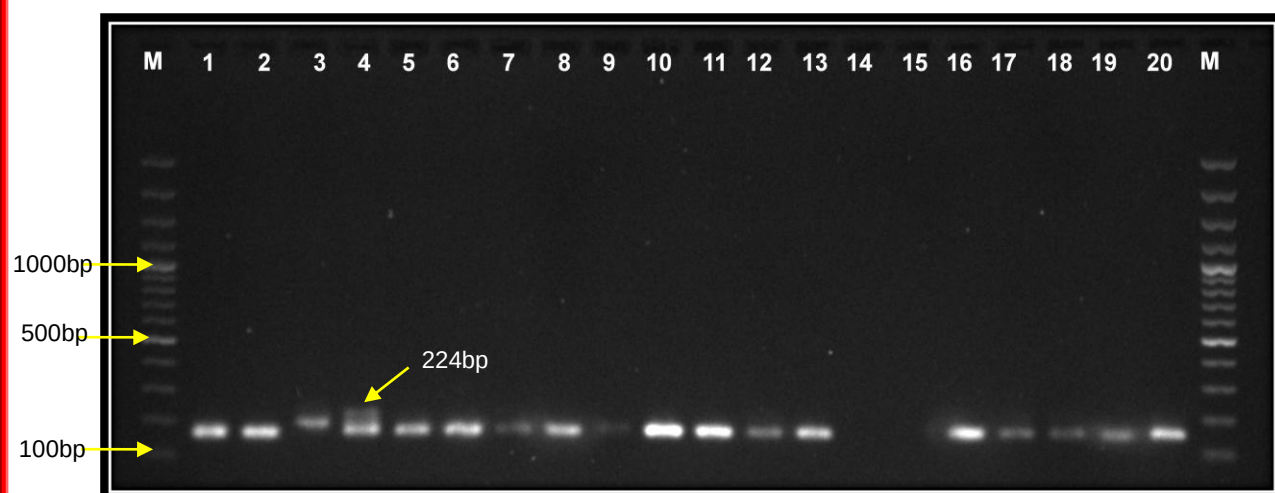


**Plate No-17. DNA fingerprinting profile generated by SSR primer-LMCH-78**

**M:-** 100+ bp ladder

**List of 20 *Annona* genotypes**

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 8.    | Balanagar        | 15.   | ACC-5                     |
| 2.    | <i>Annona reticulata</i> | 9.    | GJCA-1           | 16.   | ACC-6                     |
| 3.    | <i>Annona muricata</i>   | 10.   | Vidyanagar local | 17.   | Sindhan×Anand selection   |
| 4.    | <i>Annona atemoya</i>    | 11.   | ACC-1            | 18.   | Sindhan×Balanagar         |
| 5.    | Red sitaphal             | 12.   | ACC-2            | 19.   | Anand selection×Balanagar |
| 6.    | Anand selection          | 13.   | ACC-3            | 20.   | Balanagar×Red sitaphal    |
| 7.    | Sindhan                  | 14.   | ACC-4            |       |                           |

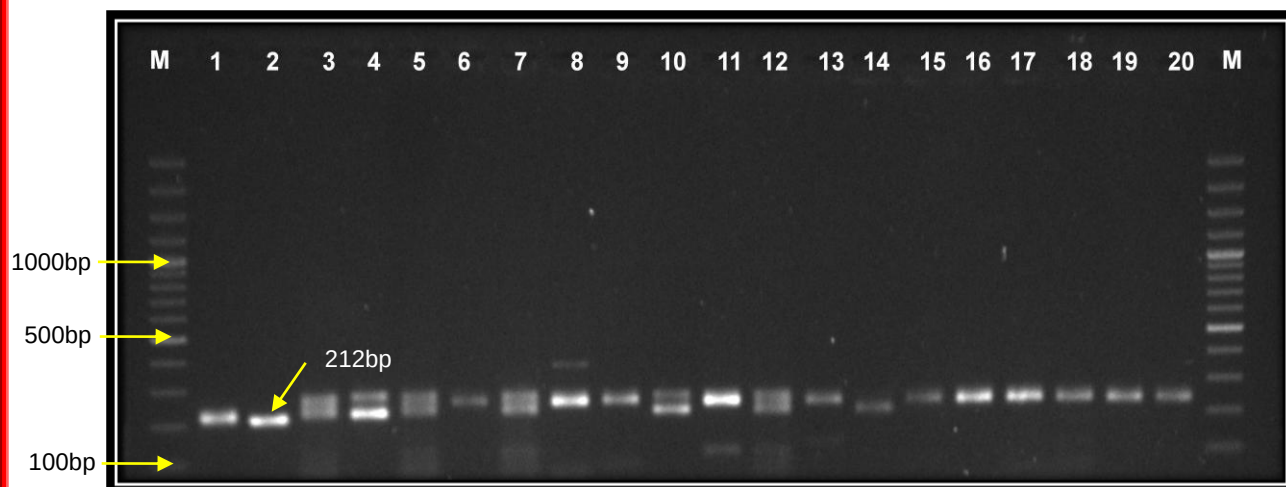


**Plate No-18. DNA fingerprinting profile generated by SSR primer-LMCH-79**

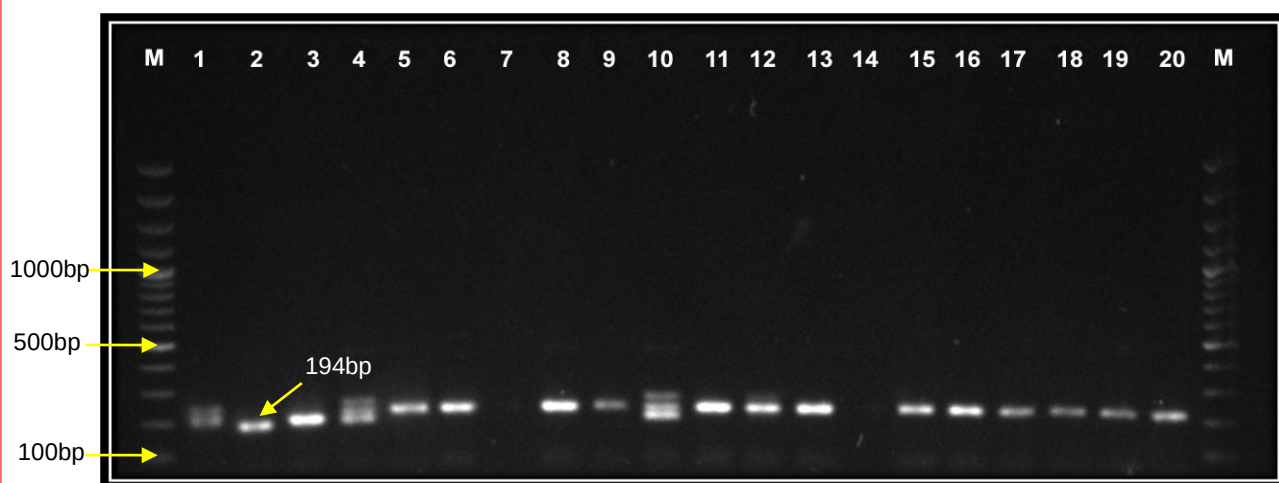


**Plate No-19. DNA fingerprinting profile generated by SSR primer-LMCH-112**

**M:-** 100+ bp ladder

**List of 20 *Annona* genotypes**

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 8.    | Balanagar        | 15.   | ACC-5                     |
| 2.    | <i>Annona reticulata</i> | 9.    | GJCA-1           | 16.   | ACC-6                     |
| 3.    | <i>Annona muricata</i>   | 10.   | Vidyanagar local | 17.   | Sindhana×Anand selection  |
| 4.    | <i>Annona atemoya</i>    | 11.   | ACC-1            | 18.   | Sindhana×Balanagar        |
| 5.    | Red sitaphal             | 12.   | ACC-2            | 19.   | Anand selection×Balanagar |
| 6.    | Anand selection          | 13.   | ACC-3            | 20.   | Balanagar×Red sitaphal    |
| 7.    | Sindhana                 | 14.   | ACC-4            |       |                           |

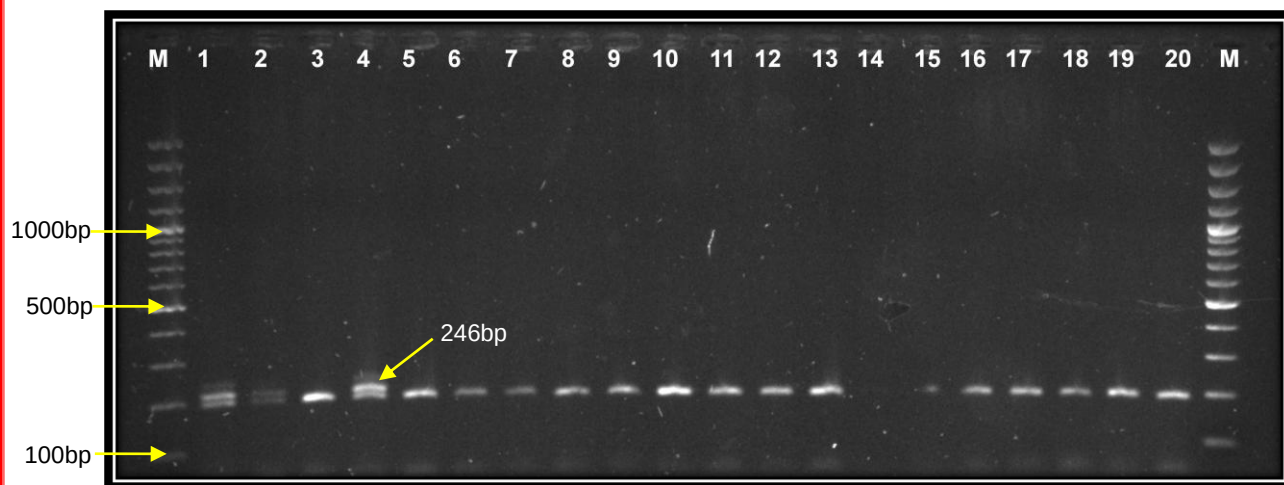


**Plate No-20. DNA fingerprinting profile generated by SSR primer-LMCH-114**

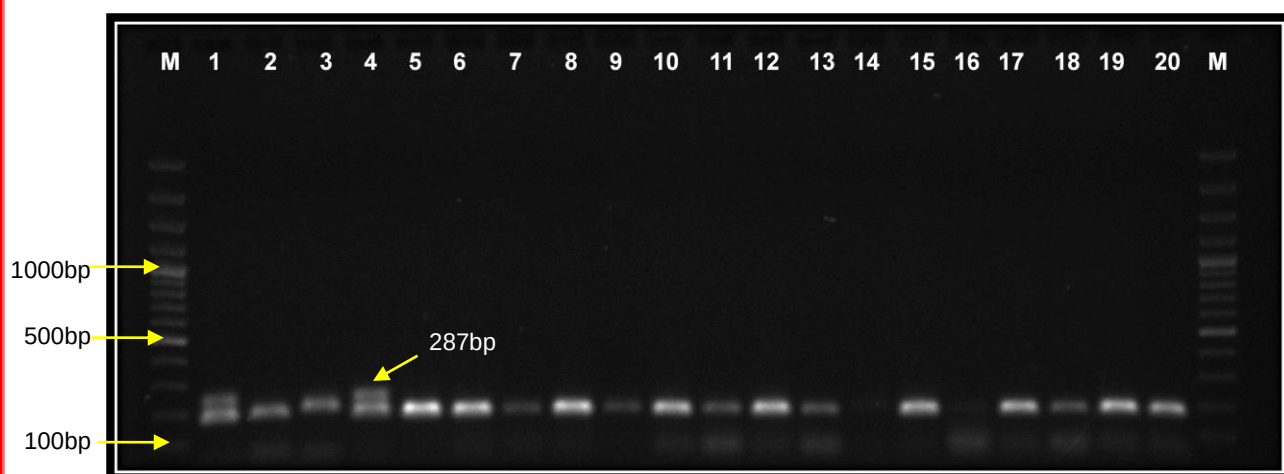


**Plate No-21. DNA fingerprinting profile generated by SSR primer-LMCH-119**

**M:-** 100+ bp ladder

**List of 20 *Annona* genotypes**

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 8.    | Balanagar        | 15.   | ACC-5                     |
| 2.    | <i>Annona reticulata</i> | 9.    | GJCA-1           | 16.   | ACC-6                     |
| 3.    | <i>Annona muricata</i>   | 10.   | Vidyanagar local | 17.   | Sindhyan×Anand selection  |
| 4.    | <i>Annona atemoya</i>    | 11.   | ACC-1            | 18.   | Sindhyan×Balanagar        |
| 5.    | Red sitaphal             | 12.   | ACC-2            | 19.   | Anand selection×Balanagar |
| 6.    | Anand selection          | 13.   | ACC-3            | 20.   | Balanagar×Red sitaphal    |
| 7.    | Sindhyan                 | 14.   | ACC-4            |       |                           |

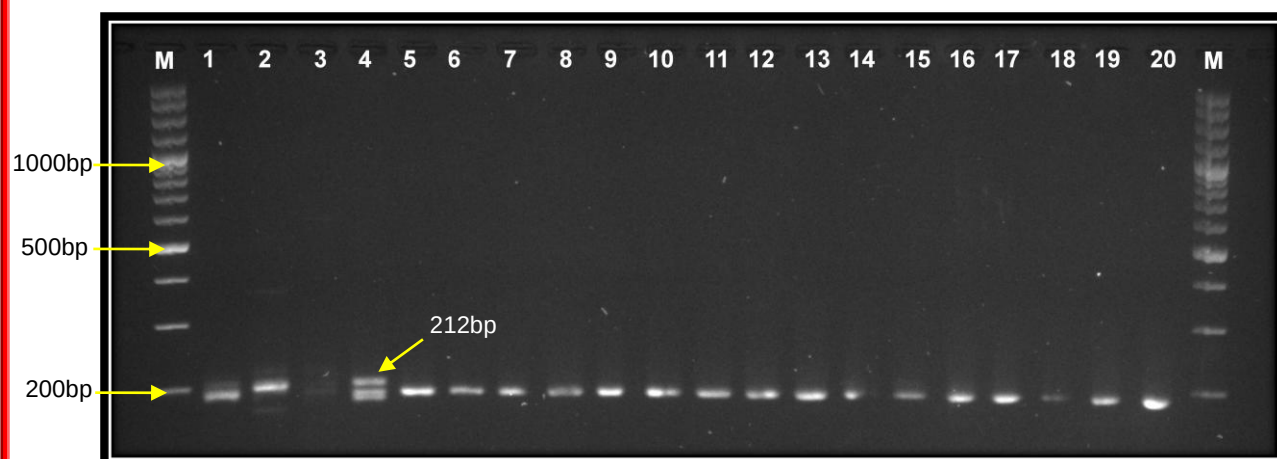


**Plate No-22. DNA fingerprinting profile generated by SSR primer-LMCH-122**

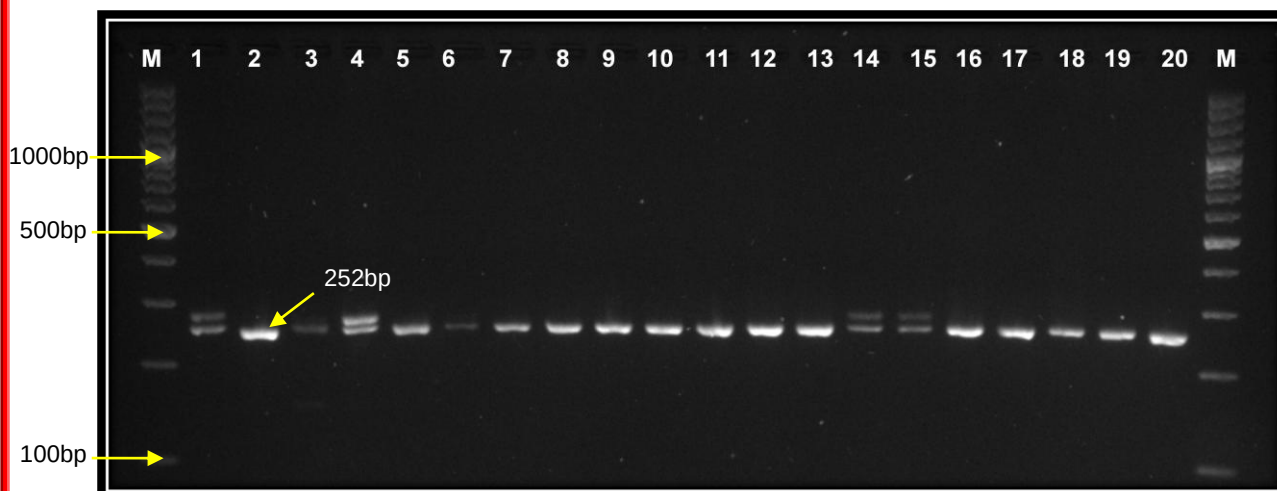


**Plate No-23. DNA fingerprinting profile generated by SSR primer-LMCH-128**

**M:-** 100+ bp ladder

**List of 20 *Annona* genotypes**

| S.No. | Genotype                 | S.No. | Genotype         | S.No. | Genotype                  |
|-------|--------------------------|-------|------------------|-------|---------------------------|
| 1.    | <i>Annona cherimola</i>  | 8.    | Balanagar        | 15.   | ACC-5                     |
| 2.    | <i>Annona reticulata</i> | 9.    | GJCA-1           | 16.   | ACC-6                     |
| 3.    | <i>Annona muricata</i>   | 10.   | Vidyanagar local | 17.   | Sindhan×Anand selection   |
| 4.    | <i>Annona atemoya</i>    | 11.   | ACC-1            | 18.   | Sindhan×Balanagar         |
| 5.    | Red sitaphal             | 12.   | ACC-2            | 19.   | Anand selection×Balanagar |
| 6.    | Anand selection          | 13.   | ACC-3            | 20.   | Balanagar×Red sitaphal    |
| 7.    | Sindhan                  | 14.   | ACC-4            |       |                           |