भारतीय नींबू (*सिट्रस मेडिका* एल0) में आनुवांषिक विभिन्नता एवम् जातिवृत्तीय संबन्धों का अध्ययन

"Genetic variability and Phylogenetic relationship studies in Indian Citron (*Citrus medica*. L)"

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DIVISION OF PLANT GENETIC RESOURCES

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"Genetic variability and phylogenetic relationship studies in Indian

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A Thesis

By

AJIT UCHOI

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C E R T I F I C A T E

This is to certify that the thesis entitled, **"Genetic variability and Phylogenetic relationship studies in Indian Citron (*Citrus medica*. L)"** submitted to the Faculty of the Post Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY IN PLANT GENETIC RESOURCES** is a record of *bona fide* research work carried out by **Mr. AJIT UCHOI, Roll No. 9874** under my guidance and supervision, and that no part of this thesis has been submitted by him for any other degree or diploma. It is further certified that all the assistance and help availed during the course of investigation as well as all sources of information have been duly acknowledged by him.

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ABBREVIATIONS

AFLP: Amplified Fragment Length Polymorphism

ASL: Above Sea Level

bp: Base pair

cpDNA: Chloroplast DNA

CTAB: Hexacetyl Trimethyl Ammonium Bromide

DDW: Double Distilled Water

dNTPs: Deoxyribonucleotide triphosphates

EDTA: Ethylene Diamine Tetra Acetic acid

EMI: Effective marker index

IBPGR: Indian Bureau of Plant Genetic Resources

IPGRI: International Plant Genetic Resources Institute

IRAP: Inter-retroelement amplified polymorphisms

ISSR: Inter Simple Sequence Repeats

ISTA: International Seed Testing Association

ITS: Internal transcribed spacer

J: Jaccard's coefficient

Kb: Kilobases

LN: Liquid nitrogen

NJ: Neighbor Joining

mg: Milligram

MI: Marker Index

ml: Milliliter

MP: Maximum Parsimony

mM : Millimolar

mw: Molecular Weight

ng: Nanogram

NTSYS: Numerical Taxonomy and Multivariate Analysis System

PCA: Principal Component Analysis

PCoA: Principle Co-ordinate Analysis

PCR: Polymerase Chain Reaction

PIC:Polymorphic information content

pmoles: Picomoles

PVP: Polyvinyl Pyrrolidone

PVS: Plant vitrification solution

rDNA: ribosomal DNA

RAPD: Random Amplified Polymorphic DNA

rbcL: Ribulose bisphosphate carboxylase/oxygenase

RFLP: Restriction Fragment Length Polymorphism

SCAR: Sequenced Characterized Amplified Region

SMC: Simple Matching Coefficient

SSR: Simple sequence Repeats

Taq : *Thermobacillus aquaticus*

TE: Tris-EDTA (buffer)

UPGMA: Unweighted Paired Group Method with Arithmetic averages

µl: Microliter

µM: Micromolar

Chapter I

Introduction

The genus *Citrus* L., sole source of the citrus fruits of commerce, belongs to the orange sub-family Aurantioideae of the family Rutaceae. *Citrus* is believed to have its primary centre of origin in south and south-east Asia, particularly in the region extending from North-East India, eastward through the Malayan Archipelago to China and Japan, and southward to Australia (Swingle and Reece, 1967; Scora, 1975; Gmitter and Hu, 1990; Mabberley, 2004).

Citrus is one of the most extensively cultivated fruit crop in the world. However, *Citrus* taxonomy and phylogeny have been difficult and controversial, mainly due to widely sexual compatibility between *Citrus* and related genera, polyembryony (adventive embryony), high frequency of bud mutations, wide dispersion and the long history of cultivation (Nicolosi *et al*., 2000; Moore, 2001).

Before the mid-1970s, *Citrus* taxonomy was mainly based on morphological and geographical data, resulting in various taxonomic systems, among which those of Swingle and Reece (1967) and Tanaka (1977) are the most widely accepted. The number of species to be recognized in *Citrus*,but the relationships among genotypes is the major problem in *Citrus* taxonomy. Swingle recognized 16 species in *Citrus*, while Tanaka documented 162 species. In the mid-1970s, an inclusive study in *Citrus* based on morphological and biochemical characteristics (Scora, 1975; Barrett and Rhodes, 1976) proposed and revealed that cultivated *Citrus* comprises only three basic species, i.e., *C. medica* (Citron), *C. reticulata* (Mandarin) and *C. maxima* (Pummelo). The other genotypes namely orange, grapefruit, lemon and lime were originated from one or more generations of hybridization among these true species or between them and species of the subgenus *Papeda* or closely related genera.

Citrus medica commonly known as Citron is indigenous to India. It is commonly found in the warm valleys along the foot hills of the Himalayas from Garhwal to Sikkim ascending to 4,000 feet in the Khasi hills, the Garo Hills and also in the Western Ghats and Satpura ranges of Madhya Pradesh. The true citron is called *bira-jora* or *bakol-khowa-tenga* (*bakol* means rind, *khowa* to eat and *tenga* stands for sourness) in Assam

valley plains, *jaara-jamir* in Sylhet and Cachar, *Soh-manong* in the Khasi Hills, *Sutrung* in Bengal etc. Citron is monoembryonic in nature and is considered as one of the three basic species of *Citrus.* The Citron is mainly used for medicinal purposes:*viz*., to combat sea sickness, pulmonary troubles, intestinal ailments, and other stomach disorders. It is also used by Jews (the word for it in Hebrew is Etrog) for a religious ritual during the Feast of Tabernacles. Its fruit is widely used for making jam, pickles and preservatives (Malik *et al*., 2012).

India is rich in *Citrus* genetic resources, both in cultivated and wild species. Several earlier workers had attempted to study and classify Indian *Citrus* from botanical as well as horticultural perspectives. According to recent systematic accounts on Indian *Citrus*, Nair and Nayar (1997) included 18 taxa, which comprised 8 species under subgenus *Citrus*, 3 species under subgenus *Papeda*, and 7 species ofother indigenous *Citrus* types with a suspected hybrid origin and uncertain taxonomic affinities. However, confusion still abounds in proper identification, taxonomic disposition and circumscription of cultivated as well as wild and indigenous species/varieties of *Citrus* in India in spite of those earlier systematic reports.

1.1 Characterization

Characterization of germplasm is a basic requirement for any crop improvement programme. Vast genetic diversity of wild and semi-wild *Citrus* species of North Eastern India has minimally been used for improvement programmes due to lack of their characterization. Major limitation in undertaking characterization of most of the endangered species is insufficient germplasm availability during collections as well as no much attention is given to these aspects. Conventionally, morphological markers have been used for germplasm characterization using descriptors developed by Bioversity International (IPGRI, 1999). Increasing cost and time required in data collection and lack of knowledge of genetic control of phenotypic trait are other limitations of morphological markers leading germplasm curators towards more reliable and faster method of characterization.

Characterization by methods that directly utilize DNA could potentially address the limitations associated with morphological and biochemical markers (Kresovich, 1996).

Thus, molecular markers generated by techniques such as Random Amplified Polymorphic DNA (RAPD) (Williams *et al*. 1990; Welsh and McClelland, 1990) and Inter Simple Sequence Repeats (ISSR) are becoming increasingly important for cultivar identification (Patterson, 1996). Molecular markers detect more variation than morphological and biochemical markers and are not affected by environment and are simply inherited (Karp *et al*., 1997).

1.2 Seed Storage behaviour

Seed storage behaviour in its simplest form is measured in terms of survival and longevity of seeds under various storage conditions. Information on this is available for only about 3% of the higher plant species (Hong and Ellis, 1996). Various research groups in different countries are undertaking studies on this aspect mainly on their indigenous species. Seed storage behaviour in several cases is misinterpreted because of scanty data generated on survival and longevity of seed due to lack of complete information on physiological characteristics. Conservationist can recommend and adopt short-medium and long-term seed storage only after correct identification of seed storage behaviour. The success of *ex-situ* conservation depends on longevity of explant under storage and the ability to generate the whole explant/plantlet after retrieval from the storage conditions. Seeds of many tropical and sub-tropical plant species often of economic value have been classified as recalcitrant and intermediate (Robert, 1973). A major impediment in taking up *ex-situ* conservation of many *Citrus* species is the inadequate information available on seed storage behaviour. Seed storage behaviour is to be established for a species before developing suitable protocol for long-term conservation. It is analyzed by studying morphological and seed physiological parameters especially desiccation and freezing sensitivity along with seed longevity. Seeds on the basis of their sensitivity or tolerance to desiccation and freezing can be classified into orthodox (desiccation tolerant), intermediate (desiccation sensitive and freezing tolerant) and recalcitrant (desiccation and freezing sensitive). Seeds of *Citrus* are classified into intermediate i.e. these seeds with comparatively large size and with high moisture content at the time of shedding are variably desiccation and freezing sensitive. Long-term conservation of *Citrus* germplasm in the form of seed/embryonic axes has been found most promising due to the presence of

polyembryony (nucellar embryos) and no report of virus transmission through seeds.Most of the *Citrus* species exhibit intermediate storage behaviour and therefore, cannot be stored using conventional storage methods.

1.3 Phylogenetic relationships

Phylogeny is the study of the evolutionary relationships among group of organisms which have been discovered through molecular sequencing and morphological data matrices. It aids in proper identification, classification, taxonomy and naming of organisms. Knowledge of the genetic relatedness of *Citrus* with other genotypes is very important for establishing their classification, breeding behavior and optimum utilization of germplasm resources for various crop improvement programmes. The phylogeny and taxonomy of *Citrus* species is complex, confusing and controversial due to the genetic heterogeneity of the genus, as well as its polyembryonic nature, sexual compatibility between *Citrus* species and related genera, high frequency of bud mutations, long history of cultivation, wide dispersion and long regeneration time needed to carry out selection and recombination (Swingle, 1943; Frost and Soost, 1968; Nicolosi *et al*., 2000). Elucidating relationships, taxonomy, and diversity is important for developing breeding strategies, conserving biodiversity, and for improving breeding efficiency of species.

1.4 Cryopreservation

Cryopreservation is a long-term conservation of biological tissues at ultra-low temperature of -160 to -196˚C. Cryopreservation has also become a promising tool for long term conservation of clonally propagated germplasm resources. It facilitates management of the *in vitro* collection by minimizing the risk of both somaclonal variation and contamination of cultures. Cryopreservation, offers a long-term storage capability, maximal stability of phenotypic and genotypic behaviour of stored germplasm, and minimal storage space and maintenance requirements (Engelmann, 1997). Traditionally, *Citrus* germplasm is conserved in clonal orchards belonging to botanical gardens or to scientific institutions. Genotypes of particular value are kept in greenhouses and screen houses, where these can be more easily protected from losses due to pests, diseases and climatic hazards. However, the high costs of this traditional conservation system limit the number of accessions that can be preserved. Moreover,

many *Citrus* species are polyembryonic, meaning that several asexually-originated nucellar embryos are contained in the seed at maturation; the conservation of germplasm in traditional seed banks is difficult to apply, as many species have seeds recalcitrant to desiccation (i.e., non- or sub-orthodox seeds). Hence, conservation in liquid nitrogen (LN, at -196°C) should be considered as an alternative method for the long-term conservation of *Citrus* genetic resources.

Understanding taxonomy, phylogenetic relationships and genetic variability in *Citrus* is critical for determining genetic relationships, characterizing germplasm, controlling genetic erosion, designing sampling strategies or core collections, establishing breeding programs, and the registration of new cultivars (Herrero *et al.,* 1996). The importance of *C. medica* in the ancestry of *Citrus* has been put into question mark. Such a geographically diverse group of species functioning as the other parent of these cultigens suggests that the natural distribution of *C. medica* is not restricted to India (Bayer *et al*., 2009). Due to this long prevailing confusion of the role of *C. medica* as the basic species, there has been a critical need for studying the extent of diversity occurring in Citron group of different parts of India and their phylogenetic relationships with other closely related *Citrus* species and genera.

Till date, there is no report of genetic diversity studies and phylogenetic relationship analysis of Indian Citron with related species and genera. Thus, in the present plan of work it is proposed to emphasize on following objectives.

1. Study of genetic variability existing in Indian citron (*Citrus medica*)
2. Phylogenetic relationship studies of Indian citron in relation to other *Citrus* species
3. Cryobiological studies of seeds, embryo and embryonic axes of Indian citron

Chapter II

REVIEW OF LITERATURE

Before undertaking any study, it is important to understand and know about the various and current works status of research on any concerned topic as it gives a greater vision and ideas to carry out more meaningful experiments. In this chapter, an attempt has been made to represent all the previous work done by various researchers on citrus germplasm especially citron group.

2.1 Exploration and collection

Citrus genetic diversity was collected and assessed intensively by Tanaka (1928, 1937) and Bhattacharya and Dutta (1956) during 3rd to 5th decade of last century from North-Eastern part of India. Most of the new *Citrus* species and genotypes were identified, collected and documented from North-Eastern part of India during these studies. Since then, no significant information has been added to our knowledge about the occurrence and diversity of any additional species of *Citrus* from this important hotspot of biodiversity. However, during last 15 years recent surveys and explorations enumerated loss of diversity of some species and genotypes from various pockets where these were earlier reported (Singh and Singh, 2003; Malik *et al*., 2013). The study on genetic resources of *Citrus* from North-Eastern part of India indicated the presence of 23 species, one subspecies and 68 varieties, thus according this area with a special status known as a treasure house of *Citrus* germplasm (Sharma *et al.,* 2004;Malik *et al*., 2013). Some of the important *Citrus* species are still growing in a wild or semi-wild form in the forestlands in northeast and northwest part of India. Natural populations of these species are shrinking drastically due to large-scale deforestation to meet the land requirement for cultivation in the Himalayan region (Ahuja, 1996).

2.2 Seed physiological studies

Martins and Gonzalex-Benito(2006) reported the immediate effects of dehydration on the physiological performance of 'Cleopatra' tangerine (*C. reticulata*) seeds. Evaluations were made for the moisture level, germination, seedling emergence and root, hypocotyls

and seedling length. It was concluded that the moisture content, is favoured by dehydration to 39 percent water.

Siqueira *et al.* (2002), carried out work in order to verify the viability period of the rootstocks 'Rangpur' lime, 'Volkameriana' lemon, 'Cleopatra' mandarin and 'Swingle' citrumelo seeds stored in permeable package at 5-7˚C for 150 days. The germination in laboratory, seedling emergence in greenhouse and seed moisture content was performed after 30 days. Seed storage in those conditions reduced the speed of emergence index in the 'Rangpur' and 'Volkameriana' seeds. The germination of these rootstock seeds were not reduced when the seed moisture content was near 5 percent. There was highly significant correlation between seed water content and germination percentage of 'Cleopatra' and 'Swingle' seeds. For viability maintenance of seeds of these rootstocks, moisture content could be kept around 20 percent. The 'Rangpur' and 'Volkameriana' seeds showed higher vigour as compared with the other rootstocks, while 'Cleopatra' seeds presented lower speed of emergence index.

Role of seed coat have been studied by many workers in Citrus and it has been found that seed coat acts as a barrier to seed germination by putting physical or chemical impediments to seed germination (Monselise, 1962; King and Roberts, 1980). Seed coat has also been found to be inhibitory for germination in many species of *Citrus* (Mumford and Grout, 1979; Radhamani *et al*., 1991). The germination studies carried out before and after freezing the seeds of *C.limon* after removal of seed coat revealed the desiccation and freezing tolerance of these decoated seeds (Mumford and Grout, 1979). Similar observations were reported in earlier studies on seed germination in different *Citrus* species (Cho *et al*., 2001a, 2001b, 2002; Radhamani*et al*., 1991; Normah *et al*., 1996; Malik and Chaudhury, 2006).

2.3 Seed storage behaviour

Seed storage behaviour is to be ascertained essentially for any species before developing protocol for long term conservation of germplasm and to understand longevity and storage conditions of seeds. Seed storage behaviour has been divided into three different categories. Initially, Roberts (1973) defined two categories namely orthodox and recalcitrant. Another category of seed storage behaviour is in between

orthodox and recalcitrant which was reported in Coffee and Citrus (Hong and Ellis, 1996).

Genus *Citrus* is reported to comprise of species with variable seed storage behaviour in all three categories i.e. orthodox, intermediate and recalcitrant present in this genus. Seeds of many *Citrus* species display recalcitrant or intermediate storage behaviour and therefore, cannot be stored using conventional storage methods (King and Roberts, 1979). Seeds of many *Citrus* species have been reported to be showing orthodox storage behaviour with or without testa (King *et al*., 1981). However, Hong and Ellis (1995) categorized many species of *Citrus* under intermediate, recalcitrant and orthodox on the basis of reports available in the literature. This physiological characteristic has been utilized in studies to develop suitable protocol for long-term conservation of various *Citrus* species. Oliveira *et al.,* (2003) established a protocol for seed storage of *Poncirus trifoliata*, citrus rootstock. Seed storage behaviour of several tropical fruit species including indigenous *Citrus* species has also been reported (Malik *et al.,* 2006; 2012).
Hamilton (2009) studied the seed biology, cryopreservation and *in vitro* culture of three Australian wild *Citrus* species *viz*., *C.australisica*, *C. inodora* and*C.garrawayi* to facilitate germplasm storage. In her study, seeds of *C. australisica* and *C.inodora* exhibited orthodox seed storage behavior but *C. garrawayi* exhibited a more complex seed storage behavior, while orthodox seed storing protocol was applied for all the three species.

2.4 Genetic diversity:

Several studies have utilized various molecular markers (ISSR, RAPD, AFLP, and SSR) to fingerprint accessions, evaluate phylogenetic relationships among accessions, and examine the level of genetic diversity in *Citrus*. Many of these studies have targeted specific *Citrus* groups or sampled a few individuals of each taxon.
In *Citrus*, isozyme markers were also used for studying the genetic variability, genetic relationships and taxonomy. Scora (1975) proposed the three basic species concept (*C. medica*, *C. reticulata* and *C. maxima*) based on morphological and iso-enzymatic studies. He also proposed two areas of origin, *i.e.* tropical and sub-tropical for the subgenera *Papeda* and *Citrus*, respectively.

Herrero *et al*. (1996) studied the pattern and variation of genetic variability based on isozymes in the *Citrus* and related genera belonging to the subfamily Aurantioideae. They utilized ten enzymatic systems, which allowed distinguishing all the species. They observed high percentage of heterozygosity in the lemons and limes, indicating the origin of these species through inter-specific hybridization.

Fang and Roose,(1997) employed isozymes, RFLP and ISSR markers to classify 48 trifoliate orange (*Poncirus trifoliata*) accessions into four groups. Fang and Roose utilized ISSR markers to distinguish closely related cultivars, many of which had arisen by selection of spontaneous mutations. This study showed that ISSR markers could distinguish some (but not all) of these closely related accessions.

Federici *et al*. (1998) examined the phylogenetic relations of 88 accessions representing 45 *Citrus* species and six related genera by utilizing RFLP and RAPD markers. Overall, these previous studies demonstrated that molecular markers are powerful tools for elucidating genetic diversity, determining parentage, and revealing phylogenetic relationships among various *Citrus* species.

Elisiario *et al*. (1999) used RAPD markers for revealing the genetic relationship among *Citrus* species (Sweet oranges, Lemons, Grapefruits, Clementine and several other Mandarin biotypes). RAPD analysis was carried out using 36 decamer primers that revealed 289 bands, 48.4% of which were polymorphic. The RAPD technique, though discriminating among all the species and distinguishing among the mandarin varieties, including Carvalhais and Fremont, was unable to discriminate among the different varieties within the remaining *Citrus* species (biotypes).

Nicolosi *et al*. (2000) used RAPD, SCAR and cpDNA markers to elucidate phylogenetic relationships and genetic origins of hybrids in 36 accessions of *Citrus* and one accession from each of four related genera.

Breto *et al*. (2001) examined the variability of 24 Clementine (*C. reticulata*) accessions by utilizing ISSR, RAPD and AFLP markers.

Gulsen and Roose (2001) utilized ISSR, SSR, and isozymes to assess diversity, phylogenetic relationships, and parentage in lemon (*C. limon*) accessions and related taxa, and found low genetic variation among the lemon accessions.

Abkenar and Isshiki (2003) used RAPD markers to evaluate the genetic similarity and interrelationship among 31 acid citrus species and cultivars, including sour oranges (six accessions); "Yuzu" (four accessions) and its relatives (21 accessions). Out of the 60 decamer primers screened for the study, 27 were selected which produced 108 markers; out of which 76 were polymorphic. Species or cultivar-specific RAPD markers were also found. A dendrogram constructed based on genetic distance implied that sour oranges were found to be very distinct from "Yuzu" and its relatives. "Yuzu" accessions were very closely linked to each other, however; for the other specimens' genetic polymorphism could easily be detected by RAPDs and the genetic variation between accessions was quite high which revealed their different origins. In this study, some RAPDs allowed the distinction of very close cultivars, for instance "Kabosu" from "aka Kabosu".

Oliveira and Radmann (2005) assessed genetic similarity in citrus cultivars using iso-enzymatic markers. Out of 30 alleles, 16 were polymorphic generated by 10 isoenzyme system. These cultivars showed high genetic similarity (>72.5%) and clustered into five main groups sweet orange, 'Clemenules' and 'Marisol' mandarins, 'Nova', 'Ortanique' and Okitsu' Satsuma mandarin.

Campos *et al*. (2005) evaluated and characterized sixty three Mandarin (*Citrus* spp) cultivars using morphological and AFLP markers. In this study, twenty quantitative and ten qualitative morphological traits from leaves, flowers and fruits were evaluated. The Mse + CAG plus Eco + ACA, and Mse + AGG AFLP primers showed the best combinations and generated 109 bands with 86% polymorphism. Both morphological and molecular markers showed a high degree of variation among the accessions indicating an important source of genetic diversity.

Golein *et al*. (2005) used SSR markers to evaluate the genetic variability of 8 sweet oranges and 6 mandarin accessions. In total, 52 putative alleles were detected using 7 primer pairs. Microsatellite markers discriminated variation observed within mandarins, but low variation observed in sweet oranges. The majority of sweet orange accessions showed a narrow genetic base suggesting that the observed morphological polymorphisms within the group must be associated with somatic mutations which were not exactly detected by these molecular markers.

Pasquale *et al*. (2006) selected five clones of sour orange (*C. aurantium*) for morphological differences and characterized by genetic and leaf volatiles analysis. The genetic studies were undertaken using ISSR and RAPD molecular markers, while the leaf essential oil patterns were obtained by chromatographic and mass spectrometric determination. Results showedthe reasonably similar information can be achieved from the two techniques, supporting each other in characterization studies.

Pang *et al.* (2007) reported the use of RAPD markers solely or in combination with AFLP markers to study the phylogenetic relationship within *Citrus* and its relatives. Wei (2007) used IRAP and REMAP markers to estimate phylogenetic relationship among 24 *Citrus* cultivars.

Baig *et al*. (2009) used RAPD markers to evaluate genetic similarity and interrelationship among 18 citrus cultivars, including 13 species and 5 hybrids. Out of 40 decamer primers screened, 25 were selected which produced 250 markers; of which 231 were polymorphic and some species or cultivar specific RAPD markers. UPGMA dendrogram clearly separated Jatti-Khatti from all major clusters at a similarity coefficient of 0.61. Two sweet orange cultivars, Jaffa and Blood red, showed maximum genetic similarity (82%). The Jatti-Khatti and King Mandarin were found to be genetically most diverse. The genetic variation between cultivars was quite high and revealed their different origins.

Genetic diversity in Iranian citrus varieties was studied by Jannati *et al.* (2009) using microsatellite markers. In this study, fifteen SSR primers were used to study the level of polymorphism among 23 citrus genotypes and four natural hybrids or bud mutations. The cluster analysis of the citrus genotypes with SSR markers resulted in 2 cluster groups: Group A consisted of Yuzo and *Poncirus*. Group B: which were further sub-grouped into three groups (i) genus *Fortunella sp* (ii) Mandarin subgroup: *C. reticulata, C. sinensis* (Pineapple, Washington Navel), Natural types (Siahvaraz, Shalmahaleh, Moallemkoh and Kotra 4 hybrids) and (iii) *C. aurantifolia, C. aurantium, C. limon, C. grandis* and *C. medica*. Microsatellite analysis clustered citron and sour orange but these taxa were quiet distant from *Fortunella* species.

SRAPs markers have been utilized for genetic diversity and phylogenetic relationship studies in *Citrus* and related genera (Uzuan *et al*. 2009).

Zerihum*et al*. (2009) characterized 12*Citrus*genotypes which belong to 4 species namely sweet orange, mandarin, grapefruit, lemon and one hybrid of *citrus*, citrange hybrid-troyer (*C. sinensis* x *C. trifoliata*)using 25 SSR markers. In this study, SSR markers showed a great potential of differentiating closely related *citrus* cultivars with a huge genetic diversity.

Amar *et al.* (2011) used a combination of SSR, SRAP and CAPS-SNP markers for genetic diversity of *Citrus* germplasm collection. Biswas *et al.* (2011) also used AFLP, SSAP, SAMPL and SSR markers for a comparative analysis of genetic diversity in *Citrus* germplasm collection.

Shrestha *et al*. (2012) studied the genetic diversity of 62 acid lime (*C. aurantifolia*) landraces from Nepal using 12 SSR markers. The average genetic similarity level among the 62 accessions was found to be 0.77 and separated five major cluster groups. The average polymorphic information content (PIC) value was found to be 0.50. This study clearly demonstrated SSR as informative markers for assessing the genetic diversity of acid lime landraces.

Abedinpour *et al*. (2014) recently evaluated the genetic diversity in *Citrus* genotypes usingInter-retroelement Amplified Polymorphisms(IRAP) molecular markers. Genetic diversity among 29 citrus genotypes was evaluated using 5 primers, which generated 49 polymorphic bands. Out of the 5 primers used, IRAP-1 and IRAP-2 was found to produce maximum and minimum polymorphic bands, respectively. Based on polymorphic bands, the range of similarity value was found to be between 0.34-0.90 with an average value of 0.65. Based on cluster analysis, the citrus genotypes were divided into five separate groups. The lowest similarity index value of 0.34 belonged to Dansy mandarin and Pummelo, and the highest similarity index value of 0.90 belonged to an unknown natural types of G74 and Siavaraz orange 3 (G6). Pummelo and mandarin were confirmed as true species of citrus in distinct cluster in this study.

Al-Anbari *et al*. (2014) studied genetic diversity of citrus in Iraq using RAPD markers. Genetic relationship among 16 genotypes of citrus cultivated in Iraq using 20 primers were used for the study. Based on their studies, the citrus was found to be classified into two main groups, where the first group consisted of Citron (*C. medica)* and its hybrids (lime and lemon). Whereas, the second group consisted of the remaining genotypes

which were further sub-clustered into three sub-groups. The first group consisted of sour orange (*C. aurantium*), sweet orange (*C. sinensis*) and grapefruit (*C. paradisi*). The second group consisted of Mandarin (*C. reticulata*) and third group consisted of Pummelo (*C. grandis*). This study was found to be consistent with previous studies using other molecular markers.

Genetic relationship among 17 citrus commercially important cultivars of Pakistan was studied by Naz*et al*. (2014) using RAPD markers. Out of 25 primers screened, fifteen decamer primers were selected for the study which produced 153 polymorphic bands. The dendrogram generated using UPGMA separated the 17 citrus genotypes into 4 main clusters. The genetic similarity value across all the genotypes was observed to be 0.66. In which, maximum similarity value (0.85) was shown in the two cultivars of Kinnow and seedless Kinnow (Mandarin) and two cultivars of Meiwa and Marumi (Kumquat). The genetic difference among the citrus varieties studied was found to be high indicating their distinct origin.

Sankar *et al*. (2014) used RAPD markers to study the genetic similarity and inter relationship among twelve sweet orange varieties. Twenty random primers were screened and selected for the study. The genetic similarity was calculated using Jaccard co-efficient which indicated maximum genetic variation between Ankalamma Gudur Sathgudi and Nadimpalli Sathgudi (0.33). This was closely followed by Ankalamma Gudur Sathgudi and Jaffa (0.35). Jaffa and Kodur Sathgudi were found to be genetically closer with value 0.84 followed by Himakuntla Sweet orange and Kodur Sathgudi (0.80).) The dendrogram generated using Unweighed pair-group method with arithmetic average (UPGMA) indicated that Sathgudi Tirupati and Ankalamma Sathgudi formed one cluster and remaining varieties formed another cluster. This was further divided into two sub-clusters where Nadimpalli Sathgudi and Valentia formed first sub-cluster and Mosambi and Red Blood Malta formed second sub-cluster. Also it was found that Jaffa and Kodur Sathgudi formed as one group and Ananthapur Sathgudi, Himakuntla Sweet Orange, Valentia Late and Hamlin Sweet orange varieties was found to be different and did not resemble any other variety.

Lamine and Mliki (2015) usedRAPD and SSR markers a comparative study of the efficiency of in assessing the genetic diversity among sour orange rootstocks. In this

study, a set of six RAPD and nine SSR markers were used in a population of 46 sour orange accessions. Various genetic parameters such as effective number of alleles, percentage of polymorphism, polymorphic information content (PIC), effective marker index (EMI) and marker index (MI) parameters were determined for both the markers in order to assess the efficiency of the two marker systems. Based on the results obtained from the study, RAPD markers were found to be significantly higher than SSR markers. Therefore, the use of SSR markers which is known to be very efficient and discriminatory did not provide supplementary information on the work. This work highlights the advantage and efficiency of using RAPD markers as an efficient tool in analyzing genetic diversity of rootstocks.

Study on genetic diversity of 47 citrons was undertaken by Ramadugu *et al*. (2015) inorder to understand their diversity and relationships within the species. Data from microsatellite markers, single nucleotide polymorphisms generated from sequences of a nuclear malate dehydrogenase gene and a chloroplastgene, rps16 were used in genetic analysis of citrons. All the three approaches used found citron to be monophyletic. The 47 citrons were clustered into three distinct groups based on population structure analysis. As per their findings, the first citron group consisted of wild, non-fingered citrons generally having locules, juice sacs and seeds within the fruit. While, the second cluster of citrons mainly consisted of fingered citrons which lacked locules, juice sacs or seeds, and some non-fingered types with smaller locules and vestigial juice sacs, but with seeds.

2.5 Phylogenetic relationship:

In *Citrus*, molecular phylogeny at various taxonomic levels has been examined in several earlier studies through application of isozymes (Herrero *et al*., 1996), RAPD and PCR-RFLP (Federici *et al.*, 1998; Abkenar *et al.,* 2004), RAPD, SCAR and PCR-RFLP (Nicolosi *et al*., 2000), AFLP (Liang *et al*., 2007; Pang *et al*., 2007), SSR (Barkley *et al*., 2006), ISSR (Shahsavar *et al.,* 2007) and sequence data analysis of coding and non-coding chloroplast DNA (cpDNA) regions (Chase *et al.,* 1999; Araujo *et al*., 2003; Morton *et al*., 2003; Bayer *et al*., 2009).

Restriction site variation of PCR – amplified cpDNA (PCR-RFLP) (Williams *et al*., 1991; Arnold *et al*., 1991) has been applied for phylogenetic reconstruction in plants at various taxonomic levels (Taberlet *et al*., 1991; Arnold *et al*., 1991; Olmstead and Palmer, 1994; Demesure *et al*., 1995; Jansen *et al*., 1998). Similarly, the *trnL* (UAA)-*trnF* (GAA) intergenic spacer of cpDNA has also been reported as a potential region for systematics and phylogenetic studies in various plant taxa, such as *Coffea* (Cros *et al*., 1998), *Doronicum* (Fernandez *et al*., 2001), *Citrus* (Araujo *et al*., 2003), *Actinidia* (Jung *et al*., 2003). Jung *et al*. (2005) evaluated the potential of the *trnL-trnF* region in analyzing the phylogeny and systematic relationships in Korean Citrus; whileYingzhi *et al*. (2007) utilized *trnL-trnF* intergenic spacer for inferring phylogeny in wild as well as cultivated mandarins in China.

Pang *et al*., (2007) studied phylogenetic relationship within *Citrus* and its related genera using AFLP markers. In this study, 29 genotypes belonging to *Citrus*, *Poncirus*, *Fortunella*, *Microcitrus*, *Eremocitrus*, *Atalantia* and *Severinia were used for AL*FP analysis. The results obtained from the study indicatedthat *Poncirus*, *Microcitrus* and *Eremocitrus* were distant from *Citrus*. A strong affiliation was found to exist between *C. halimii* and *Fortunella* and the results of the experiment did not support *C. halimii* as the fourth basic species. It further proved that *P. polyandra* is not a hybrid between trifoliate orange and *Citrus* genotypes and it deserves separate species status. Based on the dendrogram constructed, the either separation of subgenus *Papeda* and *Citrus* or the separation of subgenus *Archicitrus* or *Metacitrus* was not clearly resolved. Based on the results, *C. ichangesis* was found to be a distinct species very different from other *Citrus* genotypes, and it is improper to classify it into subgenus *Metacitrus*. *C. reticulata*, *C. maxima* and *C. medica* were found to be separated into three distinct clusters in the dendrogram.

Jena *et al*. (2009) studied the taxonomy and phylogeny of Indian *Citrus* using PCR-RFLP of the *trnD-trnT* and *rbcL*-ORF106 regions as well as sequence data analysis of *the trnL-trnF* intergenic spacer region of cpDNA.

Kyndt *et al*. (2010) analysed the phylogenetic relationship among 69 Vietnamese citrus accessions and related genera using ITS region of the rDNA. Cluster analysis based on maximum parsimony and Bayesian analysis separated the three 'true' *Citru*s species (*C.*

medica, C. maxima and *C. reticulata)* into different clusters confirming their separate identity. Besides this, it was found that *Fortunella, P. trifoliata* and *C. hystrix* clustered among the accessions of subgenus *Citrus*.

The origin and phylogeny of cultivated citrus was studied by Li and Xie (2010) using amplified fragment length polymorphism (AFLP) fingerprints, nuclear internal transcribed spacer (ITS), and three plastid DNA regions (*psbH– petB*, *trnL – trnF*, and *trnS - trnG*). Using AFLP data, chloroplast DNA and ITS sequences, molecular phylogenetic trees were constructed with the genus *Poncirus* used as outgroup. The results indicated that acid lime (*C. aurantifolia*) and lemon were derived from citron (*C. medica*) and sour orange (*C. aurantium*). Grapefruit was a hybrid that originated from a cross between pummelo (*C. grandis*) and sweet orange. Rough lemon (*C. limon*) was believed to be a probable parent of rangpur lime (*C. limonia*) and guangxi local lemon (*C. limonia*). The data obtained from the study also demonstrated that sour orange and sweet orange were hybrids of mandarin (*C. reticulata*) and pummelo, while rough (*C. limon*) lemon was a cross between citron and mandarin. From the study, C. *aurantifolia* was believed to be a species where Papedas believed to be the female parent and *C. medica* as the male.

Penjor *et al*. (2010) sequenced the *rbcL* genes of 64 accessions from 24 genera of *Citrus* relatives and analyzed them by neighbor-joining and maximum parsimony methods. Both trees supported Swingle and Reece's (1967) treatment of the subfamily Aurantioideae as monophyletic. However, the trees did not support Swingle and Reece's treatment of tribes and subtribes. The subgenera *Citrus* and *Papeda* were not clustered clearly. The analysis associated the *Fortunella* group with mandarin, *Poncirus* with *C.ichangensis*, *Severinia buxifolia* with *Atalantia ceylanica*, *Microcitrus* with *Eremocitrus* and *C. micrantha*, and *Hesperethusa acrenulata* with *Citropsis*. Furthermore, *Atalantia* species showed polytomy.

Froelicher *et al*. (2011) conducted a phylogenic study using four polymorphic primers on 77 genotypes representing the diversity of *Citrus* and two related genera. Seven mitotypes were identified using this study. Six mitotypes (*Poncirus, Fortunella, C. medica, C. micrantha, C .reticulata* and *C. maxima*) were congruent with previous taxonomic investigations. The seventh mitotype enabled to distinguish an acidic

mandarin group (Cleopatra, Sunki and Shekwasha) from other mandarins and revealed a maternal relationship with *C. limonia* (Rangpur lime, Volkamer lemon) and *C. jambhiri* (Rough lemon). This mitotype contained only cultivated species used as rootstocks due to their good tolerances to abiotic stress. Their results also suggested that two species classified by Swingle and Reece (1967), *C. limon* and *C. aurantifolia*, have multiple maternal cytoplasmic origins.

The phylogenetic relationships of *Citrus* and its relatives were studied by Penjor *et al*. (2013) based on *matK* gene sequences. The chloroplast *matK* genes of 135 accessions belonging to 22 genera of Aurantioideae were sequenced and analyzed phylogenetically in this study. Based on previous studies by different authors, the subfamily Aurantioideae has been mainly classified into 2 tribes viz. Clauseneae and Citreae, and the current molecular analysis clearly discriminated Citreae from Clauseneae by using only 1 chloroplast DNA sequence. This study was found to be in accordance with previous observations on the molecular phylogeny of Aurantioideae in many aspects. They also found that ‘‘true citrus fruit trees’’ could be divided into 2 sub-clusters. In the first sub-cluster *Citrus*, *Fortunella*, and *Poncirus* were included while the other cluster consisted of *Microcitrus* and *Eremocitrus*. Based on the results obtained from the study, the author concluded that *Citrus* species can be classified into 3 clusters: a citron cluster, a pummelo cluster, and a mandarin cluster.

Amar *et al*. (2014) analyzed the phylogenetic relationships in citrus using internal transcribed spacer sequence data of ribosomal DNA (rDNA). In this study, 24 *Citrus* species and close relatives were used and were evaluated for several parameters such as nucleotide substitution (r), nucleotide diversity (p) and the estimated values of transition/transversion bias (R). The results obtained from the study indicated the presence of a wide divergence pattern of rDNA in subfamily Aurantioideae. Also Maximum Parsimony (MP) analysis inferred wide divergence pattern in the genus*Citrus*. In the study, seven strongly supported clades among the subfamily Aurantioideae were observed.

A study conducted by Kumar *et al*. (2014) identified ‘Themachi’ as a new natural wild type of citron (*C. medica*). In this study, sequences of chloroplast non-coding region *psbM-trnD* of 16 accessions of *Citrus* were used to confirm the taxonomic identity of

'Themachhi' and also to infer its phylogenetic affinities to other related *Citrus* taxa. Based on the results of the study, 'Themachhi' was found to have least genetic divergence with *C. medica* (0.013) and highest divergence was found with *C. limon* (average 0.024). All the accessions of Citrus used in the study were found to be grouped into three main clusters (*C. medica, C. reticulata* and *C. maxima* clusters) in both the MP and ML trees. This study also showed that Themachhi to be genetically very close to *C. medica* and it consistently grouped with the typical citron in all the phylogenetic trees.

Sun *et al*. (2015) recently evaluated the taxonomy and phylogeny of *Citrus* species using sequence analysis of the ITS region of nrDNA. 22 *Citrus* species having wild, domesticated, and cultivated species were used in the study. The DNA alignment of the ITS sequence showed relatively high variations of nucleotide and sequence length among these *Citrus* species viz. in ITS1 and ITS2 regions. The phylogenetic tree generated based on the ITS sequences indicated subgenera *Citrus* as monophyletic. The study found the *Citrus* species to be nearer to *Fortunella*, *Poncirus*, and *Clymenia* ascompared to *Microcitrus* and *Eremocitrus*.

2.6 Cryopreservation

Cryopreservation techniques have been extensively used for long-term conservation of various *Citrus* species and allied genera *P. trifoliata* using range of explants like zygotic embryos/embryonic axes (Normah *et al*, 1997; Radhamani and Chandel, 1992: Cho *et al*., 2001a,b, 2002; Malik and Chaudhury, 2006; Malik *et al*., 2012), shoot apices (Gonzalez-Arnao *et al*., 1998), cell suspensions (Engelmann *et al*., 1994; Sakai *et al*., 1990) and somatic embryos (Marin and Duran-vila, 1988) with varied degree of success.

Comparison between cryopreserved *Citrus* species embryos and embryos conserved at seed banking temperatures was carried out by Malik *et. al.,* (2012). The developed cryopreservation protocols were applied routinely to cryobanking of germplasm. 377 accessions of diverse citrus germplasm from field gene-banks, farmer's orchards, semi-wild and wild sources were tested for regrowth after an average of 6.3 to 8.4 years cryo-storage, between 69 and 81% of accessions per species retained ≥ 70% of the viability after desiccation. The results provided irrevocable evidence for the importance of cryopreservation for the long termcryobanking of seeds of higher plants (Malik *et al.,* 2012).

2.6.1 Air desiccation

Cryopreservation of *C. aurantifolia* seeds and embryonic axes using air desiccation (AD) was performed by Cho *et al*., 2002. The desiccation and freezing tolerance of seeds, with and without testa, and embryonic axes of *C. aurantifolia* were investigated. They observed that survival before and after cryopreservation was higher for seeds without testa irrespective of the desiccation method employed. They also observed that survival of control embryonic axes was high whatever the sucrose concentration in the pre-culture medium and the duration of the desiccation period. No survival was noted with embryonic axes after cryopreservation which had not been pre-cultured nor desiccated (Malik *et al*., 2012).

2.6.2 Vitrification

A vitrification technique used to freeze apices of a range of species is being used with variable success to freeze embryonic axes of some intermediate and recalcitrant species like Neem (Chaudhury and Malik, 1999) and Jackfruit (Thammasiri, 1999). Using this technique in *Citrus* species, a recovery rate of 62.5% was reported in *C. sinensis* and 82.5% in *C. madurensis* (Cho *et al.,* 2001b).

Cryopreservation of embryonic axes using vitrification method has been attempted by Malik and Chaudhury (2006) in two wild and endangered species of citrus. They obtained a high recovery rate of 92% and 77% for *C. macroptera* and *C. latipes*, respectively. Cho *et al*., (2002) cryopreserved the embryonic axes of*C. madurensis* by vitrification. They showed the importance of loading and treatment with vitrification solution on the survival of *C. madurensis* embryonic axes (Malik *et al*., 2012).

2.6.3 Encapsulation-dehydration

The encapsulation-dehydration technique has been reported for two *Citrus* rootstocks (*P. trifoliata* and citrange) (Engelmann *et al.,* 1994), while anencapsulation-vitrification procedure has also been developed with the sour orange (*C. aurantium*) (Sakai 2000).

The use of encapsulation-dehydration technique in the cryopreservation of *C. madurensis* embryonic axes was demonstrated by Cho *et al* (2002). Up to 57.5% survival was achieved using a standard encapsulation-dehydration protocol, which included pre-growth

of encapsulated axes for 16h in medium containing 0.8 M sucrose +1 M glycerol, desiccation of beads around to 30% moisture content (fresh weight basis) followed by rapid freezing (Malik *et al*., 2012).

Chapter III

Materials and Methods

The physiological studies including seed germination, longevity, storage behaviour, morphological, molecular characterization and phylogenetic studies were being undertaken at cryolaboratory of Tissue Culture and Cryopreservation (TCCU) laboratory and Division of Genomic Resources at ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR), New Delhi, India.

3.1 Plant material

Exploration and collection trips have been undertaken for survey and collection of the germplasm of *C.medica* in the form of fruits from different parts of India mainly from the North-Eastern states of India.

Figure 3.1: Diversity of various Indian citron (*C. medica*) cultivars

3.2 Collection of plant materials

Specific exploration and collection missions were undertaken to different parts of India particularly the North-Eastern region of India viz., Assam, Arunachal Pradesh, Meghalaya, Nagaland, Sikkim and North-Western states of Himachal Pradesh, Uttarakhand and Punjab for the survey and collection of *C. medica* germplasm. A total of forty six representative accessions of diverse citron cultivars were collected from wild, semi wild and cultivated conditions for undertakingvarious experimentalstudies (Table3.1). Collections were mostly made following selective sampling strategy, where samples collected from single plant was given an indigenous collection number (IC number) and treated as individual accession. Leaf and fruit samples of each accession were taken for confirmation of taxonomic identity, morphological and molecular characterization and DNA extraction. Detailed information of each accession was recorded in passport database of ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR), New Delhi, India.

The fruits were cut open and seeds were extracted by peeling off the transparent membrane of closely packed carpel segments and removing the mucilage by washing in warm water. Seeds were surface dried by spreading on the blotting sheets at room temperature to remove excess water. Number of seeds per fruits, seed shape, size (length x breadth), colour and seed weight was recorded.

Freshly extracted seeds were used for estimation of moisture content and viability. While for further experiments seeds were treated with fungicide. Seeds to be used for storage experiments were coated with 1g Thiram powder per kg seeds and kept in refrigerator until further use for various experiments.

Endocarp was removed before the initiation of any of the experiments.

Figure 3.2: Map of India showing different collection sites of Citron accessions for the present study

3.3 Moisture content

Moisture content of seeds was determined using low constant temperature oven method by drying at 103±2˚C for 17hrs (ISTA, 1985). A part from 5-10 seeds in duplicate was taken for each determination and moisture content expressed on fresh weight basis. The moisture content in seeds was calculated by using the formula.

Moisture content= B – C × 100 / B – A

Where, **A** = weight of empty weighing bottle

B = weight of bottle + fresh seeds

C = weight of bottle + oven dried seeds

3.3.1 Seed germination

Seeds were sterilized by treating with 0.1% mercuric chloride for 10-12 minutes followed by three rinses in sterile deionised water. Freshly extracted, desiccated and stored seeds were placed for germination in plastic Petri plates (11cm diameter) lined with filter paper disc as substrata at 27±2˚C with 16/8h light/dark photoperiod. Germination was scored as the extrusion of the radical.

Germination index (G.I) = n/d

Where, n= no. of seedling emerging on day'd'

d = day after plating

3.4 Estimation of seedling vigour and vigour index

The 30 days old seedlings after germination were scored for root and shoot length. The vigour index is calculated as the product of seedling vigour (total of root and shoot length) and germination percentage.

Vigour index = Germination % × Seedling length

3.5 Morphological characterization

Thirty seven discrete morphological traits which include 12 quantitative and 25 qualitative characters were selected based on the descriptors developed by IPGRI (Presently Bioversity International) (IPGRI, 1999). The characters were converted into bistates and multistates (interval) code. Standardization of morphological data was done based on YBAR option with thensoftware NTSYS ver. 2.10e (Rohlf, 2000). A pairwise similarity matrix was generated using Simple Matching coefficient and an NJ tree was constructed based on Euclidean distance methodwith the same software.

Table 3.1: List of Indian citron accessions used for morphological analysis

Sl. No.	Col. No.	IC No.	Cultivar/local name	Place of Collection
1	MD-475	470376	Tayum	Arunachal Pradesh
2	MD-467	311349	Tayum	Arunachal Pradesh
3	MDY-71	395400	Citron	Punjab
4	MD-07/115	558139	Themachi	Meghalaya
5	MD-7/123	558147	Themachi	Meghalaya
6	MD-10/49	586997	Bemberia	Sikkim
7	MD-10/62	587010	Bemberia	Sikkim
8	MS-10/21	558147	Themachhi	Meghalaya
9	MS-10/41	587029	Themachhi	Meghalaya
10	MS-10/58	558139	Themachhi	Meghalaya
11	MSA-11/18		Citron	Himachal Pradesh
12	MD-11/48	591406	Pati Jora	Assam
13	MD-11/49	591407	Bira Jora	Assam
14	MD-11/52	591410	Soh-mondong	Assam
15	MD-11/63	591421	Citron	Assam
16	MD-11/64	591422	Gondhraj	Assam
17	MD-11/65	591424	Pongam Citron	Assam
18	MD-11/66	591425	Holong tenga	Assam
19	MD-11/73	591433	Citron	Assam
20	MD-11/74	591442	Bore Tenga	Assam
21	MD-11/84	591443	Jora Tenga	Assam
22	MD-11/85	591444	Citron	Assam
23	MD-11/99	591458	Chonchuno	Nagaland

3.6 Molecular characterization:

For molecular studies by DNA markers, a total of 46 accessions were taken in total. Young leaves were randomly removed from mother plant from which the fruits were collected and stored at -20˚C until DNA extraction. Leaves were also removed from seedlings grown in petriplates, but from those seeds in which all embryos had developed into seedlings. The leaves were removed under aseptic condition and wrapped in aluminum foil after labelling and kept at -20˚C.

Table 3.2: Details of 46 accessions of *Citrus medica* collected from different parts of India, used for RAPD, ISSR and SSR analysis.

Sl no.	Collector number	Cultivar name	Source	Latitude	Longitude	IC number	Biological status
1	MD-17	Bemberia	Sakhu,East Sikkim	27.13	88.30	586997	Semi-wild
2	MD-22	Bemberia	Lingzo, South Sikkim	27.03	88.25	587002	Semi-wild
3	MD-30	Bemberia	Darjeeling, West Bengal	27.04	88.39	587010	Semi-wild
4	MS-15	Themachi	East Garo Hills, Meghalaya	25.29	90.19	558139	Wild
5	MS-21	Themachi	East Garo Hills, Meghalaya	25.29	90.19	558140	Wild
6	MS-41	Themachi	East Garo Hills, Meghalaya	25.29	90.19	587029	Wild
7	MS-42	Themachi	East Garo Hills, Meghalaya	25.35	90.16	558147	Wild
8	MS-58	Themachi	East Garo Hills, Meghalaya	25.30	90.20	-	Wild
9	MD-154	Themachi	East Garo Hills, Meghalaya	25.30	90.20	417222	Wild
10	MD-176	Themachi	South Garo Hills, Meghalaya	25.44	90.82	417243	Wild
11	IC-344926	Themachi	East Garo Hills, Meghalaya	25.30	90.20	344926	Wild
12	MD-11/48	Pati jora	Tinsukia, Assam	27.31	95.21	591406	Cultivated
13	MD-11/49	Bira jora	Tinsukia, Assam	27.31	95.21	591407	Cultivated
14	MD-11/52	Soh-mondong	Tinsukia, Assam	27.31	95.21	591410	Cultivated
15	MD-11/63	Citron	Tinsukia, Assam	27.31	95.21	591421	Cultivated
16	MD-11/64	Gondharaj	Tinsukia, Assam	27.31	95.21	591422	Cultivated
17	MD-11/65	Pongam-Citron	Tinsukia, Assam	27.31	95.21	591424	Cultivated
18	MD-11/66	Holong Tenga	Tinsukia, Assam	27.31	95.21	591425	Cultivated
19	MD-11/73	Bore Tenga	Tinsukia, Assam	27.31	95.21	591433	Cultivated
20	MD-11/83	Jora Tenga	Joygukhawa, Assam	27.32	97.23	591442	Cultivated
21	MD-11/84	Citron	Dibru Saikhowa,Assam	27.35	95.21	591443	Natural wild
22	MD-11/99	Chonchuno	Tesophenyu, Nagaland	25.58	94.13	591458	Wild
23	MD-202	Etrog	Upper Subansiri, Arunachal Pradesh	27.56	94.20	417268	Wild
24	CITRON-AP	Tayum	Upper Subansiri, Arunachal Pradesh	27.56	94.20	583259	Wild
25	A-I	Tayum	West Siang, Arunachal Pradesh	28.05	94.42	-	Wild
26	CITRON-	Tayum	West Siang	28.05	94.42	583270	Wild

	I		,Arunachal Pradesh				
27	MSA-11/18	Etrog	Hamirpur, Himachal Pradesh	31.50	76.30	-	Cultivated
28	MBS-11	Citron	Abohar Field gene bank, Punjab	30.21	74.22	-	Cultivated
29	MD-61	Citron	Abohar field gene bank , Punjab	30.21	74.22	311379	Cultivated
30	MDY-71	Citron	Abohar field gene bank, Punjab	30.21	74.22	395400	Cultivated
31	MD-467	Citron	Abohar field gene bank, Punjab	30.21	74.22	311349	Cultivated
32	N/SKV/1418	Citron	Bahraich, Uttar Pradesh	28.35	79.42	219067	Cultivated
33	P/N/SKV-1418	Citron	Bahraich, Uttar Pradesh	28.35	79.42	319067	Cultivated
34	MAU-01	Citron	Bhowali research station, Uttarakhand	29.20	79.30	-	Cultivated
35	MAU-02	Citron	Bhowali research station, Uttarakhand	29.20	79.30	-	Cultivated
36	MAU-03	Citron	Bhowali research station, Uttarakhand	29.20	79.30	-	Cultivated
37	MAU-04	Citron	Bhowali research station, Uttarakhand	29.20	79.30	-	Cultivated
38	MAU-05	Citron	Bhowali research station, Uttarakhand	29.20	79.30	-	Cultivated
39	MAU-06	Citron	Bhowali research station, Uttarakhand	29.20	79.30	-	Cultivated
40	IC-318908	Citron	Bhowali, Uttarakhand	29.20	79.30	318908	Cultivated
41	IC-319049	Citron	Bhowali, Uttarakhand	29.20	79.30	319049	Cultivated
42	IC-320210	Citron	Bhowali, Uttarakhand	29.20	79.30	320210	Cultivated
43	IPS/PNB/319	Citron	Bhowali, Uttarakhand	29.20	79.30	326648	Cultivated
44	IC-202113	Citron	Bhowali, Uttarakhand	29.20	79.30	202113	Cultivated
45	IC-285406	Citron	Bhowali, Uttarakhand	29.20	79.30	285406	Cultivated
46	A1N1	Citron	Bhowali, Uttarakhand	29.20	79.30	-	Cultivated

3.6.1 DNA extraction

Extraction of total DNA was carried out as per the modified protocol of Doyle and Doyle (1990). Solutions, reagents and instruments used in this experiment are described in Appendix I.

About 100-200 mg of leaf sample was ground to fine powder in pre-cooled pestle and mortar in liquid nitrogen. Due care was taken to prevent the thawing of the material. The powdered material was immediately transferred to a 45 ml autoclaved centrifuge tube having 20 ml of preheated (65˚C for 30 minutes) CTAB extraction buffer. The mixture was incubated at 65˚C in water bath for one hour with shaking at regular interval.

An equal volume of chloroform: isoamyl alcohol (24:1) was added to the centrifuge tube. The contents were mixed by gentle swirling for 15 to 20 minutes. The tubes were centrifuged at 12,000 rpm for 20 minutes at 25˚C. The supernatant was transferred to the fresh centrifuge tubes and equal volume of chilled isopropanol was added. The contents were mixed by gentle inversions. The tubes were left for overnight at 4˚C. It was then centrifuged at 8000 rpm for 6 minutes. The DNA was spooled out carefully and kept in 1.5ml eppendorf tubes, which were then spun at 10,000 rpm for 10 minutes. The aqueous part was decanted. Ethanol (70 percent) was added to the pellet of DNA. The tubes were spun at 10,000 rpm for 10 minutes at 25˚C. The aqueous part was decanted and the pellet was dried free of ethanol. The DNA pellet was then dissolved in 10:1 Tris EDTA buffer.

3.6.2 DNA purification

RNase (20mg/ml) was added to the crude DNA at a concentration of 40 ng/ml of DNA and was incubated for an hour at 37˚C. Equal volume of phenol: chloroform: isoamyl alcohol(25:24:1)mixture was added to the DNAsolution and mixed by swirling for five minutes. The eppendorf tubes were centrifuged at 10,000 rpm for five minutes and supernatant was collected in a fresh tube. This was followed by two extractions with chloroform: isoamyl alcohol (24:1). The purified DNA was precipitated by adding 2.5 times chilled isopropanol. After mixing properly, it was incubated at -20˚C for 1 hour. DNA was pelleted by centrifugation at 12,000 rpm for 5 minutes and washed with 70% ethanol. It was then air dried and dissolved in 1X TE buffer.

3.6.3 DNA quantification

For DNA quantification, the isolated DNA was run in 1.8% agarose gel.Agarose gel was prepared by melting 1.8 g of agarose in 100 ml of 1X TAE. Upon cooling ethidium bromide was added at the rate of 0.5 µg/ml and the contents were gently swirled and poured in a gel casting tray with a properly placed comb. Polymerization of gel was allowed for 30 minutes, after which the comb was taken out carefully without any damage to the wells. The gel was transferred to an electrophoresis unit having appropriate quantity of 1X TAE.

2 µl DNA sample and 3 µl loading dye (Appendix I) was added to each veil of the agarose gel. A known amount of uncut λ phage DNA control was loaded in first three wells, e.g. 50 ng, 100 ng and 200 ng**.** The gel was run at 80 V for one hour. An approximate estimate of quantity of DNA was obtained by comparing the band intensities of the marker fragment and the sample DNA.

3.6.4 Dilution of DNA samples

Part of each DNA sample was diluted with sterilized distilled water to yield a working concentration of 10ng/µl. The diluted samples were stored at 4˚C for immediateuse, while the original undiluted samples were kept for long term storage at -20˚C.

3.7 RAPD-PCR amplification

The RAPD primers of Operon Technologies Alameda, CA, USA were used for molecular analysis. A total of 50 primers were screened with *C. medica,* of which 17 primers were selected for final profiling based on banding patterns and reproducibility (Table 2). PCR-amplification was carried out in a total reaction volume of 25 µl, containing 10 mM Tris–HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 1.0 U *Taq* DNA polymerase (G-Biosciences, India), 0.2 mM of dNTP each, 0.2 µM of RAPD primer and 20-25 ng of DNA template. DNA amplification was carried out in a BioeR Xp thermocycler and the thermal cycler conditions for PCR reactions were an initial denaturation cycle for 2 min at 94°C, followed by 40 cycles comprising 1 min at 94°C, 1 min at 35°C and 2 min at 72°C. An additional cycle of 5 min at 72°C was used for final extension.

3.8 ISSR-PCR amplification

For ISSR-PCR optimization trials, a total of 50 primers of University of British Columbia (UBC # 9) were custom synthesized from Bioscript, India and screened in citron accessions. Out of 50, only 11 primers, gave the best amplification results with the entire DNA samples, were selected for the final ISSR-PCR analysis. PCR-amplification was carried out in 25 ml reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.0– 2.5 mM $MgCl_2$, 0.2 mM dNTP each, 1.0 U Taq DNA polymerase (G-Biosciences, India), 0.2 mM primer and 20–25 ng genomic DNA. The DNA amplification was carried out in a BioeR Xp thermocycler with reaction conditions programmed as initial pre-denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at optimized temperature for 1 min, and extension at 72°C for 2 min. A final 7 min extension at 72°C followed the completion of 35 cycles. The RAPD and ISSR experiments were repeated three times.

3.9 SSR-PCR amplification

Sixteen SSR primer pairs were used in the analysis of the 46 DNA samples. SSR amplification was performed as described by Barkley *et al*., 2006 with minor modifications. The PCR-amplification was carried out in 25 ml reactionvolume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.0–2.5 mM MgCl2, 0.2 mM dNTP each, 1.0 U Taq DNA polymerase(G-Biosciences, India), 0.2 mM primer and 20–25 ng genomicDNA. The DNA amplification was carried out in a BioeR Xp thermocyclerwith reaction conditions programmed as initial pre-denaturation at 94°C for 3 minfollowed by 35 cycles of denaturation at 94°Cfor 1 min, annealingat optimized temperature for 1 min, and extension at 72°C for 1 min. A final 7 min extension at 72°C followed the completion of35 cycles.

3.9 Gel preparation

Appropriate amount of agarose was weighed, dissolved in 1X TAE to get final concentration of 1.8% agarose gel. It was melted in a microwave oven. Molten agarose was cooled down to 50˚C and ethidium bromide (2.5 µl/100 ml) was added. It was then poured into a gel mould having comb in place. After polymerization, the comb was taken

out carefully. The gel was transferred to an electrophoresis apparatus having appropriate quantity of 1X TAE buffer.

For SSR analysis, the amplification products were separated byelectrophoresis on 3% agarose gels stained with Gel view stain (G-Biosciences, India) at 120 V for 3 h and bands were visualized and documentedunder UV Gel Doc System (Mega Biosystematica, U.K).

3.10 Electrophoresis

PCR products having 2 µl loading dye was loaded in the wells of agarose gel. 12 µl Gene ruler (1 kb) was loaded in the first lane of each gel to determine the size of identified bands. PCR product from the mother plant DNA was added in the second lane and subsequent wells with the amplified products of seedlings. Electrophoresis was carried out at 120 V for 2 hours and photographed using gel doc, Mega Biosystematica, U.K.

3.11 Data analysis

The amplification products were scored as 1 and 0 for present and absent bands or alleles, respectively. Molecular weight of the amplified bands was estimated by using 1kb DNA ladder (G-Biosciences, India) as standard. The data matrix was analyzed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS) ver. 2.10e software.

Polymorphism Information Content (PIC) value, which is an index of the usefulness of each primer for diversity analysis, was determined as described by Smith *et al*.,(1997).

$$PIC = 1 - \sum fi^2$$

Where, $fi$is the frequency of the i^{th} allele.

A pair wise similarity matrix of all the accessions were estimated based on Jaccard's coefficient and a dendrogram was generated based on the unweighted pair group method for arithmetic mean (UPGMA) using the same software. Two-way Mantel test for goodness of fit for UPGMA cluster to the binary data and principal co-ordinate analysis (PCOA) were also performed using the NTSYS ver. 2.01e software. The binary data were also used to calculate genetic diversity parameters like observed number of alleles

per locus (na), effective number of alleles per locus (ne),total heterozygosity (Ht) and Shannon's information index (I) using the software POPGENE 1.31. Considering the uneven distribution of the target taxon in the study sites, and the asymmetry in the number of sampled genotypes from different localities, the genetic diversity parameters were calculated using the single population approach under the assumption of Hardy Weinberg Equilibrium.

3.12 Phylogenetic relationship studies

3.12.1 PCR amplification

Two regions of cpDNA (*rbcL* and *matK*) were amplified from each of the 23 accessions via PCR. The primers used for polymerase chain reaction amplification of the *rbcL* gene, were *rbcL* 1-1-F (aF) (5′ -ATGTCACCACAAACAGAGACTAAAGC-3′) and *rbcL* NN3-2 R (cR) (5′ -GCAGCAGCTAGTTCCGGGCTCCA-3′) (Bayer *et al*., 2009). For *matK* gene, the primers used were *matK*- 5 ′ *trnK* spacer *matK* 6(5′ -TGGGTTGCTAACTCAATGG-3′) and *matK*- 5 ′ *trnK* spacer *matK* 5 ′ R (5′ -GCATAAATATAYTCCYGAAARATAAGTGG-3′) (Bayer *et al*., 2009). DNA amplification was carried out in a BioeR Xp thermocycler and the concentration of PCR components was optimized for amplification: 10 mMTris (pH 8.3), 50 mM KCl, 0.2 mM dNTP each, 2.5 mM MgCl2, 1 U Taq DNA polymerase, 10 pmol primer each and 50 ng genomic DNA in 50 µl final reaction volume. The PCR was programmed as pre-denaturation at 94˚C for 3 min, 35 cycles of denaturation at 94˚C for 1 min, annealing at 57˚C for 1 min, extension at 72˚C for 1 min and final extension at 72˚C for 7 min. The total PCR products were electrophoresed in 0.8% low melting agarose gel (G Biosciences) at 120 V for 3 h, and bands were visualized in UV gel doc system (Mega Biosystematica, U.K).

3.12.2 Sequence analysis of *rbcL* and *matK* gene for phylogenetic analysis

Twenty three representatives, including one accession of *P. trifoliata* as out-group taxon, were used for comparison of *rbcL* and *matK* gene sequences. PCR products were excised and purified using Clean HiMedia kit. The yield of purified DNA was quantified using UV spectrophotometer. Eluted PCR products were sequenced using an Applied Biosystems Automated Sequencer (Model 3730, version 3.1) using both forward and

reverse primer. Sequences of 23 accessions of citrus including one out-group were annotated.

The identity of sequences was confirmed through a BLASTn search in NCBI data base (Altschul *et al*., 1997). The sequences were aligned using Clustal-W program (Higgins *et al*., 1994) with the default settings. Phylogenetic analysis was carried out in MEGA 5 software (Tamura *et al*., 2011). Pair-wise sequence divergence rates between accessions were calculated using Maximum Composite Likelihood method (Tamura *et al*., 2004). Phylogeny reconstruction was carried out using Maximum Parsimony (MP) and Neighbor Joining (NJ) methods. MP tree was constructed using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates), while NJ tree was obtained using the Maximum Composite Likelihood criterion. In MP analysis all the characters were assigned equal weights at all nucleotide positions (Fietch, 1971). In the MP and NJ analyses, all positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). Support values of the internal branches of MP and NJ trees were evaluated through boot strap method (500 replicates) (Felsenstein, 1985).

Table 3.3: List of *Citrus* species/biotype used for cpDNA sequence analysis

Sl. No.	Accession no.	Taxon identity	Common name/cultivar name	Locality	IC number	Latitude	Longitude	Biological status
1.	Citron-AP	*Citrus medica* L.	Tayum	West Siang, Arunachal pradesh	583270	28.05	94.42	Wild
2.	MD-66	*C. medica* L.	Holong Tenga	Tinsukia, Assam	591425	27.31	95.21	Cultivated
3.	MD-99	*C. medica* L.	Chonchuno	Kohima, Nagaland	591458	25.58	94.43	Wild
4.	MSA-18	*C. medica* L.	Citron	Hamirpur, Himachal Pradesh	-	31.50	76.30	Cultivated
5.	MD-22	*C. medica* L.	Bemberia	Lingzo, Sikkim	-	27.03	88.25	Semi-wild
6.	MS-58	*C. medica* L.	Themachi	East Garo Hills, Meghalaya	-	25.29	90.19	Wild
7.	MD-11/39	*C. limon* (L.) Burm.f.	Assam lemon	Tinsukia, Assam	591397	27.31	95.21	Cultivated
8.	MSA-04	*C.aurantifolia* (Christm.) Swingle	Sour lime	Hamirpur, Himachal Pradesh	593850	31.43	76.34	Cultivated
9.	MD-11/91	*C. limmettoides* Tanaka	Sweet lime	Mokokchong, Nagaland	591450	26.23	94.35	Cultivated
10.	MD-11/95	*C. limonia* Osbeck	Rangpur lime	Mokokchong, Nagaland	591454	26.22	94.27	Cultivated
11.	MD-11/57	*C. jambhiri* Lush.	Rough lemon	Tinsukia, Assam	591415	27.31	95.21	Cultivated
12.	MSA-14	*C. karna* Raf.	Karna khatta	Hamirpur, Himachal Pradesh	593859	31.44	76.36	cultivated
13.	MSA-10	*C. reticulata* Blanco	Mandarin	Hamirpur, Himachal pradesh	593855	31.42	76.40	Cultivated

14.	MSA-30	*C. maxima* (Burm.) Merr.	Pummelo	Kangra, Himachal Pradesh	593871	32.10	76.22	Cultivated
15.	MD-452	*C. sinensis* (L.) Osbeck	Sweet Orange	Abohar, Punjab	470365	30.21	74.22	Cultivated
16.	MD-07/132	*C. aurantium* L.	Bamsim	East Garo Hills, Meghalaya	558156	25.30	90.20	Cultivated
17.	S-44	*C. indica* Tanaka	Memang Narang	East Garo Hills, Meghalaya	558128	25.29	90.19	Wild
18.	S-51	*C. indica* Tanaka	Memang Narang	East Garo Hills, Meghalaya	558134	25.29	90.19	Wild
19.	MS-10	*C. latipes* (Swingle) Tanaka	Khasi papeda	East Khasi Hills, Meghalaya	587026	25.25	91.47	Wild
20.	MS-14	*C. latipes* (Swingle) Tanaka	Khasi papeda	East Khasi Hills, Meghalaya	587027	25.19	91.43	Wild
21.	MD-08/203	*C. macroptera* Montr.	Satkara	Kolasib, Mizoram	568595	24.12	92.40	Wild
22.	MD-08/210	*C. macroptera* Montr.	Hatkora	Kolasib, Mizoram	568602	24.11	92.41	Wild
23.	MD-101	*C. ichangensis* Swingle	Ketsa chupfu	Kohima, Nagaland	591461	25.37	94.01	Wild
24.	AB	*Poncirus trifoliata*	Trifoliate orange	East Garo Hills, Meghalaya	505932	25.29	90.19	Wild

3.13 Cryopreservation

Cryopreservation of embryonic axes was undertaken using three cryotechniques *viz*. air desiccation followed by fast freezing, vitrification and encapsulation-dehydration.

3.12.1. Excision of embryonic axes

Decoated seeds (embryos) were sterilized using sodium hypochlorite (2-2.7%) for 10-15 minutes. Embryos and embryonic axes being deep seated generally do not carry any infection. Hence, 8-10 min treatment with a sterilant was sufficient. The concentration of the disinfectant and the duration of the treatment were altered as required.

Embryonic axes were excided from the embryo in sterile condition in the laminar airflow using scalpel blade. While excising embryonic axes, the time taken to handle a number of samples was keptminimum so that there are no major differences in moisture contents between consequently excised embryonic axes during the experiment.

3.12.2. Air desiccation

Excised embryonic axes (EA) were kept in batches of 20-25 in the sterile air flow of laminar flow cabinet immediately after excision. Axes were then desiccated for 1 to 3 h. the desiccation time depends upon the size of the axes, the initial and desired moisture content levels. After each desiccation interval, moisture content and viability of embryonic axes were determined. About 10-15 axes were placed in sterile 2 ml cryovials and plunged into LN. Cryovials were rewarmed in a water bath at +38˚C for 5 min. The EA were then cultured on MS medium with 1 mgl^{-1} each of 6-benzylaminopurine and naphthalene acetic acid, 1 gl^{-1} charcoal and 0.7% agar. The cultures were maintained at 25 ± 2˚C with 16 h photoperiod under light intensity of 35 $\mu Em^{-2} s^{-1}$.

3.12.3. Vitrification

This procedure involves the pre-culture of explants on media enriched with cryoprotectants, followed by treatment with cryoprotectants in vitrification solutions followed by fast freezing. For vitrification experiments, aseptically excised embryonic axes were pre-cultured on basal MS medium supplemented with 0.3M sucrose and 2M

glycerol for 16-24 h. Embryonic axes in batches of 15-25 were then transferred to 1.2 ml sterile cryovials and treated with loading solution (0.4 M sucrose, 2 M glycerol in basal MS medium) for either 20 or 40 min at 25˚C. The loading solution was replaced by plant vitrification solution 2 (PVS2; 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulphoxidel for 30 min at 25˚C. Axes were then frozen by fast freezing in liquid nitrogen. In control treatments, the PVS2 solution was removed, and the axes were treated with unloading solution (1.2 M sucrose in basal medium) for 20 min before culturing on semi-solid media. Frozen cryovials were thawed after a minimum of 24 h storage by 5 min immersion in a water bath at 38 ± 1˚C, following which the loading solution was replaced with unloading solution for 20 min. The axes were then germinated in vitro. Experiments were repeated with 15-20 embryonic axes per treatment.

3.12.4. Encapsulation

In this method alginate beads are used to encapsulate embryo/embryonic axes followed by partial desiccation before plunging in liquid nitrogen. For the encapsulation-dehydration process, aseptically excised embryonic axes were encapsulated in alginate beads by first suspending in calcium-free MS basal liquid medium containing 3% (w/v) Na-alginate. Drops of this solution, each containing one axis, were dispensed with a pipette into MS basal liquid medium supplemented with 100 mM calcium chloride. Beads were solidified by 60 min incubation at 25˚C with occasional stirring, and then pre-cultured on a rotary shaker in liquid MS medium supplemented with sucrose (0.3, 0.5 or 0.75 M) at 100 rpm for 20 and 40 h. the beads were removed from liquid medium and dehydrated at room temperature for 6 h in a laminar flow cabinet. The moisture content of the beads was determined by drying at 103 ± 2˚C for 17 h. beads were finally enclosed in 1.2 ml cryovials and fast frozen in liquid nitrogen. The cryovials were thawed in a 38 ± 1˚C water bath for 5 min, and the beads cultured *in vitro*. Experiments were repeated with 10-20 axes per treatment.

(Note: the preparation of cryoprotectants solutions and pre-treatment media for cryopreservation is given in Appendix II.)

CHAPTER IV

RESULTS

6.1 Morphological characterization

C. medica is a bushy small shrub or tree of irregular habit of growth with twigs angled and purplish when young and cylindrical at maturity, glabrous, with stout, short, single spines in the axils of the leaves. The leaves are glabrous, ellipticovate or ovatelanceolate, bluntly pointed or rounded at the tips, cuneate or rounded at the base with serrated margins. Petioles were short, wingless or narrowly margined, not clearly articulated with the leaf blade or sometime articulation absent. The inflorescences are short, fewflowered racemes; flower buds large, purplish; flowers perfect; petals 5, pinkish on the outside; stamens very numerous, 3040 or even 60; ovary large, with 1013 locules, tapering into the thick style. The fruits were large, oblong or oval, ellipsoid, surface smooth or more often rough and bumpy, mammilated apex, yellowish when ripe, with very thick rind, segments small, filled with pale greenish pulpvesicles with acid or sweetish pulp. Seeds were monoembryonic in nature, numerous in numbers, small, pointed at the base, smooth with spheroid or ovoid shape; cotyledon white and reddish chalazal cap.

Comparative analysis of 37 morphological characters examined in the 23 accessions of citron cultivars revealed moderate to significant variation among them. Maximum similarity was observed between accessions number MD07/ 115 and MD10/ 58 (0.78), while minimum similarity between MD11/49 and MD10/62 (0.11) (Table 4.2). The NJ tree (Figure 4.1), based on Euclidean distance divided the accessions into two main clusters: Cluster I consisted of 21 accessions while cluster II consisted of only two accessions. The first cluster was further subdivided into five sub-clusters, out of which accessions MD07/115 and MD10/58 are found to be closely related to each other with a similarity value of 0.78. Accession MD11/66 was found to be distinct from other accessions as it formed a separate sub-cluster.

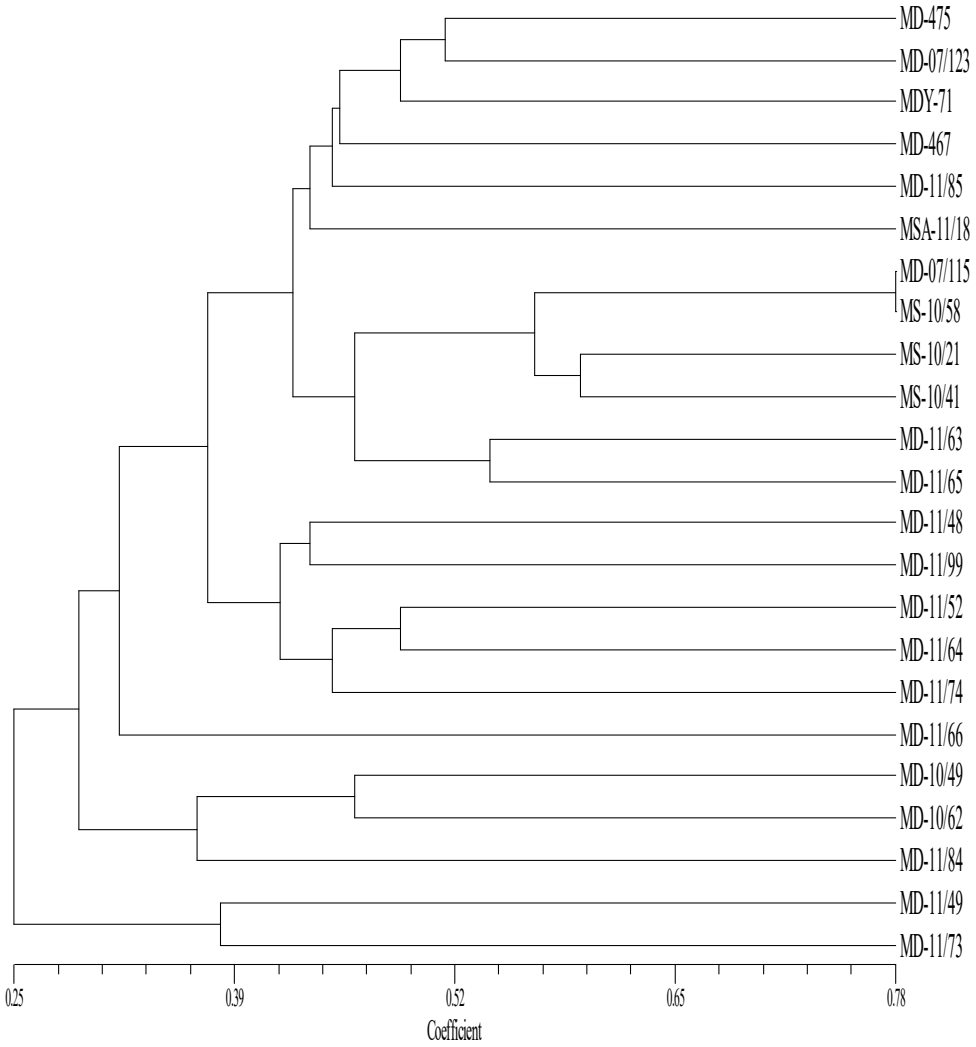

Figure 4.1: UPGMA dendrogram of 23 Indian citron accessions based on morphological analysis

Based on Mantel Z statistics the correlation coefficient (r) was estimated at 0.78. A value of 0.78 is considered a good fit of the UPGMA cluster pattern to the data. A twodimensional (2-D) plot generated from PCA showed five groups which was found to be more or less similar to the clustering pattern of the UPGMA dendrogram. The analysis gave 23 principal components out of which first ten principal components contributed 85.02 % of the total variability. The first five principal components accounted for 61.88 % of the total variation and the first three accounted for 46.02 % of the variation, in which maximum variation was contributed by first component (19.75 %) followed by

second component (14.81 %), and third component (11.47 %). The first PC was highly influenced by characteristics of the fruit morphology *viz*., fruit shape, skin colour, surface texture, rind thickness, leaf lamina margin, seed shape, chalazal spot colour, nature of oil gland and petiole wing shape (Table 4.1). In second PC, the traits contributing to the total variability were fruit weight, fruit length, width of epicarp at equatorial area, leaf thickness and petiole wing width. The third PC was influenced by fruit length, rind thickness, shape, surface texture, seed shape, seed colour, intensity of green colour of leaf blade and leaf lamina shape.

Table 4.1: PrincipalComponent Analysis (PCA) of various qualitative and quantitative characters of citron

Sl. No.	Characters	Mean	Std	Range	PC-1	PC-2	PC-3
1	Fruit weight	191.2	131.01	43.00-515.00	-0.28	0.16	0
2	Fruit diameter	60.63	17.83	35.08-89.27	0.04	-0	-0.08
3	Fruit length	83.23	23.82	43.18-145.11	0.11	0.06	0.04
4	Width of epicarp at equatorial area	1.94	0.57	0.57-3.22	0.28	0.09	0.1
5	Fruit rind thickness	7.59	2.22	2.67-10.39	0.36	0.09	0.14
6	No. of segments per fruit	10.49	0.89	8.67-13.00	0.29	-0.1	0.02
7	Taste of juice TSS	7.11	1.45	4.0-10.50	0.29	-0.2	-0.08
8	No. of seed/fruit	33.63	32.71	2.0-100.00	-0.5	-0.6	0.06
9	10 Seed weight	0.69	0.13	0.28-1.05	0.24	-0.1	0
10	Leaf lamina length	87.54	16.73	61.72-111.83	0.24	-0	0.01
11	Leaf lamina width	48.84	7.05	34.20-60.90	0.27	-0	0.03
12	Leaf thickness	0.52	0.24	0.11-1.01	0.19	0.07	0
13	Fruit shape	1.87	0.34	1.00-2.00	0.36	-0.1	0.07
14	Shape of fruit base	3.09	0.85	2.00-5.00	0.21	-0	-0.04
15	Shape of fruit apex	1.04	0.21	1.00-2.00	0.3	-0.1	-0.07
16	Fruit skin colour	2.39	2.02	1.00-6.00	0.45	-0.1	-0.47
17	Fruit surface texture	1.69	1.02	1.00-4.00	0.44	0.06	0.19
18	Adherence of albedo to pulp	1.91	0.42	5.00-7.00	0.31	-0	0.02
19	Nature of Oil glands	2.04	0.29	1.00-2.00	0.36	-0.1	-0.02

20	Density of oil glands on fruit surface	1.26	0.47	1.00-3.00	0.28	-0.2	-0.02
21	Oil gland size on fruit surface	1.69	0.45	1.00-2.00	0.24	0.02	-0.07
22	Albedo colour	7.83	1.49	1.00-6.00	0.17	-0.7	-0.06
23	Pulp colour	4.57	4.81	1.00-12.00	-0.15	-0.3	-0.27
24	Pulp firmnes	4.78	0.84	3.00-5.00	0.31	-0	0.07
25	Seed shape	1.3	2.41	2.00-9.00	0.43	0.04	0.23
26	Seed surface	2.43	0.47	1.00-2.00	0.07	-0.1	0.02
27	Seed colour	5.96	1.64	1.00-6.00	0.01	-0.2	0.1
28	Colour of cotyledons	6.09	3.78	1.00-10.00	-0.16	-0.4	-0.09
29	Chalazal spot colour	4.83	1.24	4.00-9.00	0.37	-0.1	-0.02
30	Juice content in endocarp	1.69	1.47	3.00-7.00	0.1	-0.1	-0.09
31	Intensity of green colour of leaf blade	1.96	0.76	1.00-3.00	0.15	0.01	0.15
32	Leaf lamina shape	2.97	0.77	1.00-5.00	0.22	-0.1	0.19
33	Leaf lamina margin	3.87	1.63	1.00-5.00	0.33	-0	-0.18
34	Leaf apex	3.85	1.09	3.00-6.00	0.2	-0.1	-0.04
35	Absence/ presence of petiole wings	0.21	0.42	0.00-1.00	0.26	0	-1.61
36	Petiole wing width	0.73	1.48	0.00-5.00	0.24	0.08	-1.7
37	Petiole wing shape	0.87	1.18	0.00-4.00	0.68	-0	-0.56

Table 4.2: Genetic similarity coefficient among different accessions of citron

	MD-475	MD-467	MDY-71	MD-07/115	MD-07/123	MD-10/49	MD-10/62	MS-10/21	MS-10/41	MS-10/58	MSA-11/18	MD-11/48	MD-11/49	MD-11/52	MD-11/63	MD-11/64	MD-11/65	MD-11/66	MD-11/73	MD-11/74	MD-11/84	MD-11/85	MD-11/99
MD-475	1.00																						
MD-467	0.49	1.00																					
MDY-71	0.46	0.46	1.00																				
MD-07/115	0.46	0.46	0.49	1.00																			
MD-07/123	0.51	0.41	0.51	0.51	1.00																		
MD-10/49	0.41	0.41	0.32	0.27	0.38	1.00																	
MD-10/62	0.35	0.22	0.32	0.35	0.32	0.46	1.00																
MS-10/21	0.41	0.49	0.41	0.54	0.51	0.32	0.38	1.00															
MS-10/41	0.46	0.41	0.32	0.49	0.43	0.35	0.43	0.59	1.00														
MS-10/58	0.41	0.38	0.41	0.78	0.51	0.27	0.43	0.65	0.59	1.00													
MSA-11/18	0.46	0.43	0.46	0.41	0.41	0.38	0.27	0.38	0.35	0.38	1.00												
MD-11/48	0.38	0.35	0.32	0.35	0.32	0.27	0.16	0.41	0.41	0.35	0.41	1.00											
MD-11/49	0.22	0.22	0.22	0.22	0.24	0.22	0.11	0.22	0.16	0.19	0.19	0.32	1.00										
MD-11/52	0.41	0.41	0.32	0.41	0.30	0.27	0.19	0.41	0.38	0.41	0.41	0.46	0.22	1.00									
MD-11/63	0.41	0.41	0.41	0.51	0.43	0.24	0.27	0.49	0.43	0.51	0.38	0.35	0.24	0.41	1.00								
MD-11/64	0.41	0.38	0.35	0.43	0.30	0.24	0.27	0.32	0.30	0.38	0.38	0.43	0.19	0.49	0.41	1.00							
MD-11/65	0.43	0.38	0.35	0.51	0.41	0.24	0.19	0.41	0.38	0.43	0.35	0.43	0.30	0.51	0.54	0.51	1.00						
MD-11/66	0.24	0.22	0.30	0.38	0.19	0.14	0.24	0.27	0.27	0.32	0.32	0.35	0.14	0.35	0.35	0.43	0.43	1.00					
MD-11/73	0.27	0.30	0.32	0.27	0.30	0.38	0.27	0.35	0.32	0.30	0.30	0.32	0.38	0.30	0.32	0.22	0.27	0.16	1.00				
MD-11/74	0.27	0.41	0.38	0.32	0.24	0.27	0.24	0.32	0.27	0.27	0.38	0.38	0.22	0.46	0.35	0.43	0.38	0.32	0.32	1.00			
MD-11/84	0.24	0.27	0.27	0.24	0.27	0.38	0.35	0.35	0.32	0.30	0.35	0.30	0.14	0.30	0.32	0.32	0.27	0.16	0.30	0.43	1.00		
MD-11/85	0.46	0.43	0.46	0.49	0.43	0.32	0.27	0.43	0.41	0.43	0.41	0.43	0.30	0.35	0.49	0.41	0.43	0.38	0.38	0.35	0.22	1.00	
MD-11/99	0.35	0.43	0.32	0.38	0.32	0.30	0.24	0.43	0.35	0.38	0.38	0.43	0.19	0.41	0.43	0.41	0.35	0.27	0.30	0.41	0.35	0.38	1.00

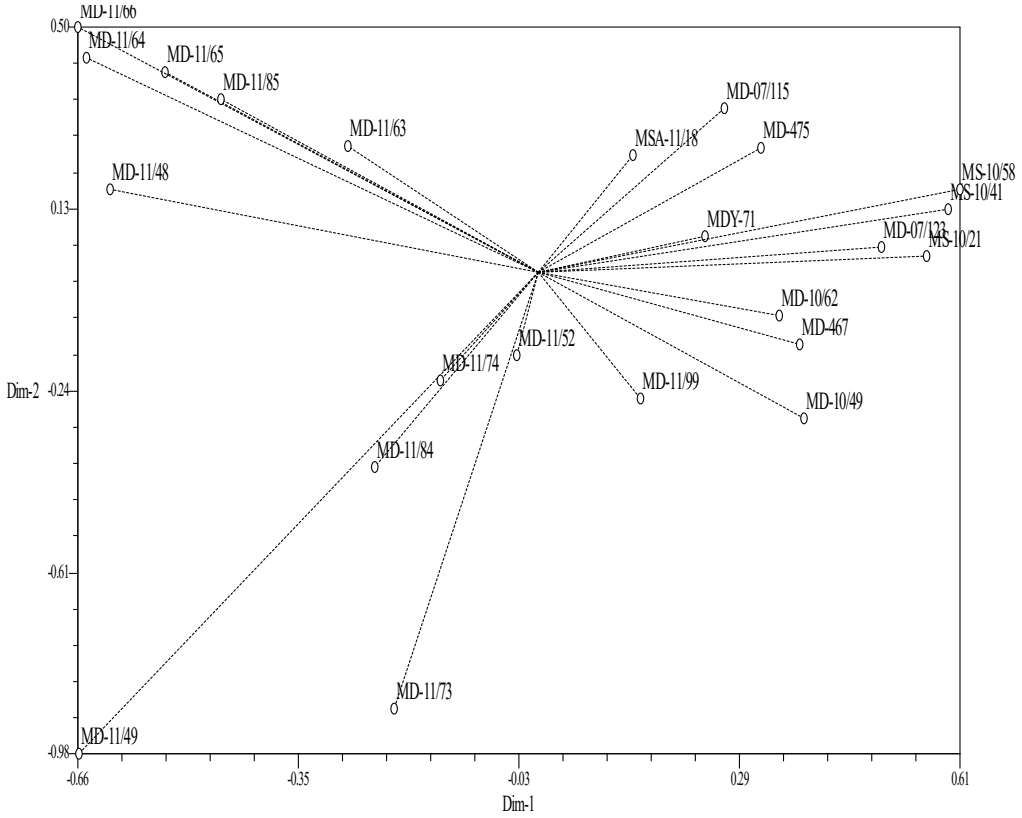

Figure 4.2: 2D diagram of 23 Indian Citron accessions based on morphological traits

6.2 Genetic diversity and relationships

6.2.1 RAPD analysis

A total of 213 bands were amplified with 17 RAPD primers in 46 accessions of *C. medica,* of which 175 bands (82%) were polymorphic (Table 4.3). The total number of amplified bands per primer varied from 5 to 18 (average 12.5), while number of polymorphic bands ranged from 3 to 15 (average 10.29) bands per primer. A representative gel profile generated using the primer OPD-02 is given in figure 4.3. The approximate size of amplified fragments ranged from 220–2000bp. The maximum number of bands (18) was generated by OPD-02 and OPD-20 primers, while OPF-01 primer amplified the minimum number of bands (5). The maximum polymorphic bands (15) were obtained with the primer OPD-20 and minimum bands (3) were generated with

primer OPF-01. PIC value of each primer varied from 0.163 (OPF-03) to 0.379 (OPU-05) with an average of 0.297. Some of the RAPD primers were able to generate accession specific unique fragment in ten different accessions (Table 4.4).

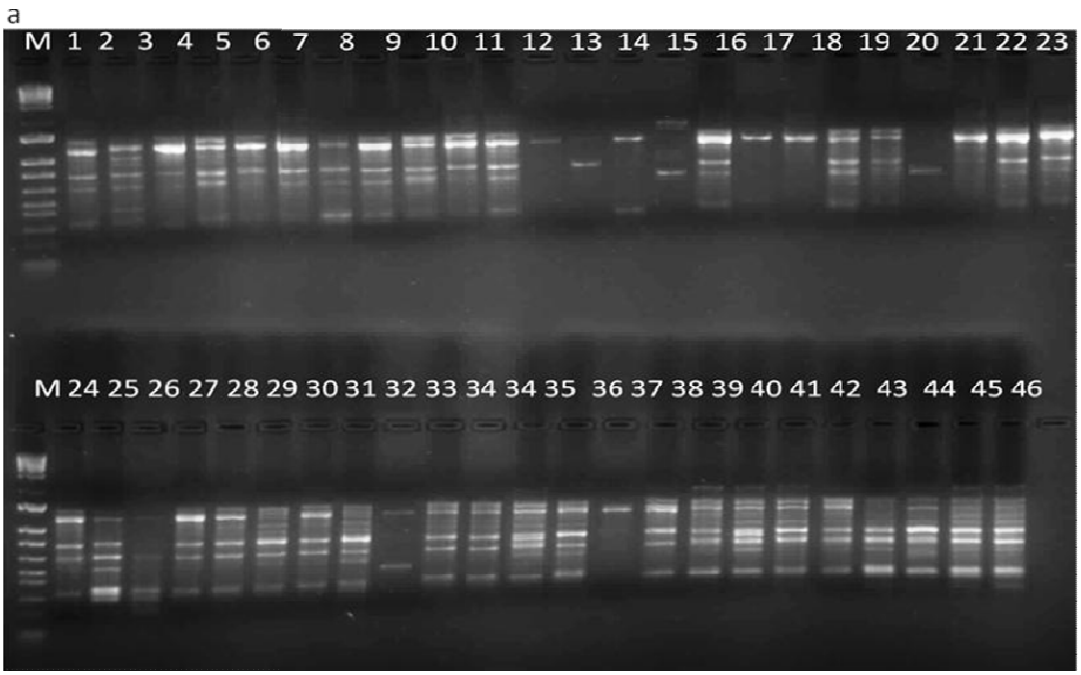

Figure 4.3: RAPD gel profile of primer OPD-02. The numbers from 1-46 are showing the Citron accessions used for the study

Table 4.3: Details of amplified bands generated by 17 RAPD primers in 46 accessions of *C. medica*

Sl. No.	Primer code	Sequence 5'-3'	Total no. of bands	PB[a]	PPB[b]	PIC[c]	Amplified fragment length
1.	OPA-04	AATCGGGCTG	15	13	87	0.3433	355-995
2.	OPB-12	CCTTGACGCA	14	13	93	0.3220	220-936
3.	OPC-08	TGGACCGGTG	9	7	78	0.3337	296-905
4.	OPD-02	GGACCCAACC	18	14	78	0.2741	328-942
5.	OPD-03	GTCGCCGTCA	15	12	80	0.2986	300-1005
6.	OPD-11	AGCGCCATTG	12	12	100	0.3057	242-820
7.	OPD-15	CATCCGTGCT	10	8	80	0.3205	275-882
8.	OPD-20	ACCCGGTCAC	18	15	83	0.3068	295-880
9.	OPF-01	ACGGATCCTG	5	3	60	0.2043	496-715
10.	OPF-02	GAGGATCCCT	11	8	73	0.3303	290-770
11.	OPF-03	CCTGATCACC	10	8	80	0.1630	320-700

12.	OPF- 16	GGAGTACTGG	15	12	80	0.3170	225-895
13.	OPU-01	ACGGACGTCA	11	9	82	0.2445	298-1200
14.	OPU-02	CTGAGGTCTC	15	12	80	0.3191	332-1498
15.	OPU-05	TTGGCGGCCT	14	11	79	0.3793	364-958
16.	OPU-08	GGCGAAGGTT	10	8	80	0.3202	355-2000
17.	OPU-14	TGGGTCCCTC	11	10	91	0.2590	580-1350
		Total	213	175	82 %	0.2965	

aTotal Polymorphic Bands, **b** Percentage of Polymorphic Bands, **c** Polymorphic Information Content (Average)

A pair wise similarity value among all the 46 accessions of *C. medica* ranged from 0.49 to 0.96 with an average of 0.73. Clustering pattern of *C. medica* revealed that all the accessions from different localities grouped on the basis of genetic relationship despite their geographical distribution. All accessions were separated into two main clusters in the UPGMA dendrogram (Figure 4.4). The first cluster comprised of 23 accessions in which two accessions namely MD-17 and MD-22 clustered distinctly with other accessions. The second cluster also consisted of 23 accessions where accessions no. A1N1 and IC-285406 was found to be closely related with a similarity value of 0.96.

Table 4.4:Unique bands for RAPD markers identified in the present study

Sl. No.	Primers	Number of bands	Band size (bp)	Accession no.
1.	OPA-04	1	980	MD-11/49
2.	OPC-08	2	450	IC-219067
			900	MAU-06
3.	OPD-11	1	670	CITRON-AP
4.	OPD-20	3	820	MD-22
			590	MD-17
			420	MD-22
5.	OPF-03	3	700	IC-319049
			470	MD-22
			450	MD-22
6.	OPF-16	3	750	MSA-18

			270	MD-22
			240	MD-17
7.	OPU-01	1	1150	MD-17
8.	OPU-02	1	750	IC-202113
9.	OPU-05	1	700	MD-154
10.	OPU-08	1	1800	IC-202113
11.	OPU-14	1	1330	IC-202113
	Total	18		

Mantel test revealed high and significant co-phenetic correlation coefficient (r = 0.96), which shows a very good fit of UPGMA dendrogram to the data.A 2D plot generated from principle co-ordinate analysis (PCoA) of RAPD data also supported the clustering pattern of the UPGMA dendrogram (Figure 4.5). First and second principal components accounted for 31.03 and 9.71 %, respectively, of the total variation. The values of mean observed number of alleles (na), mean effective number of alleles (ne), total heterozygosity (Ht), and Shannon's information index (I) were 1.991, 1.418, 0.257 and 0.400, respectively.

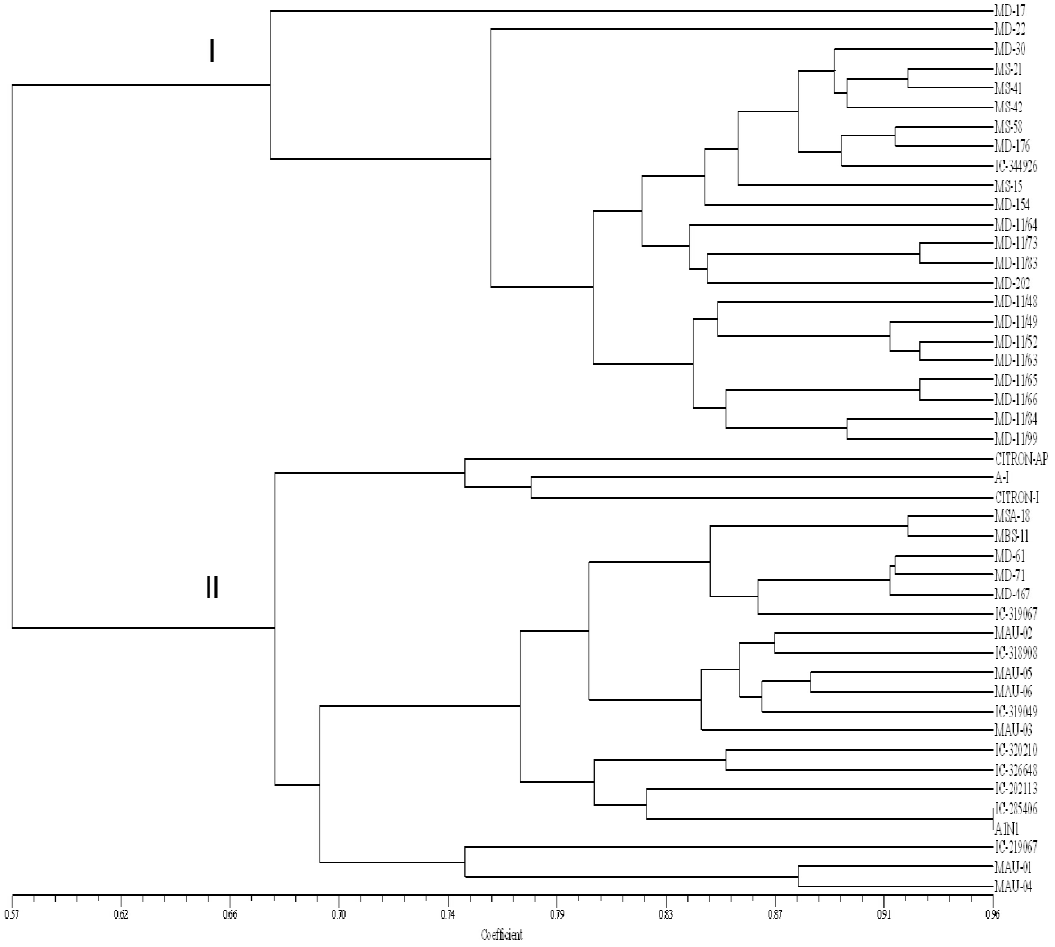

Figure 4.4: UPGMA dendrogram of 46 Indian citron accessions based on RAPD marker

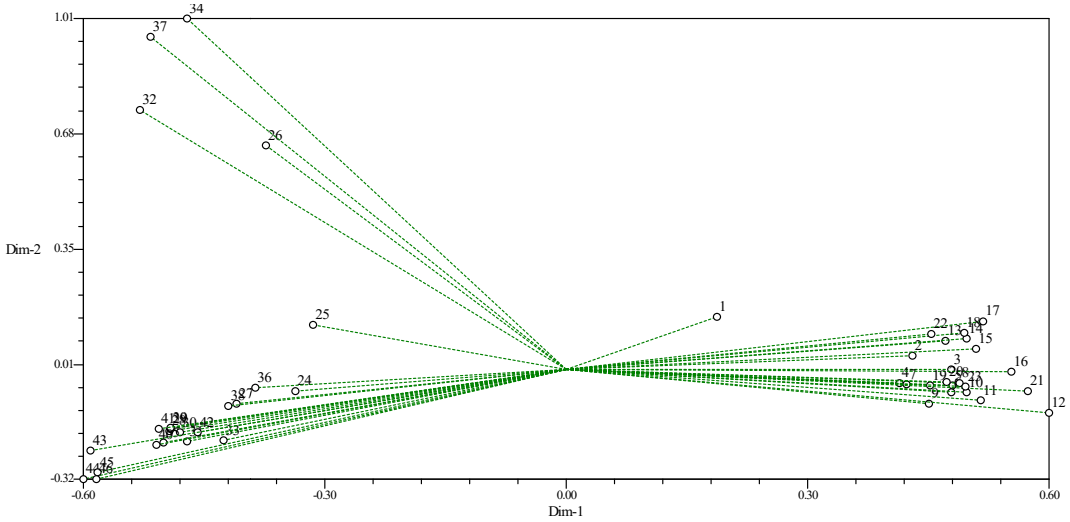

Figure 4.5: 2D plot of 23 Indian citron accessions based RAPD data

6.2.2 ISSR analysis

A total of 105 bands were scored by 11 primers among the 46 accessions of *C. medica*, of which 77 bands were polymorphic (73%) with an average of 7 polymorphic bands per primer (Table 4.5). The fragment size ranged from 140bp to 1350bp. A representative ISSR gel profile is shown in figure 4.6. Each of the 11 primers amplified a range of 5 to 14 bands with an average of 9.5 bands per primer. The primer UBC-808 generated the maximum number of bands (14), whereas UBC-880 amplified the minimum number of bands (5). Primer UBC-808 generated the highest number of 11 polymorphic bands. PIC value of each primer varied from 0.139 to 0.407 (average 0.332). Some of the primers were able to generate unique marker bands in certain accessions (Table 4.6). A pairwise similarity value among all the accessions of *C. medica* ranged from 0.38 to 0.99 with an average value of 0.69. The UPGMA dendrogram placed all the 46 accessions in two main separate clusters(Figure4.7). The first cluster consisted of eight accessions. The second cluster consisted of 38 accessions which were further sub-divided into three sub-clusters. Within this cluster, MSA-17 and MBS-11 accessions were closely related to each other with a similarity value of 0.99.

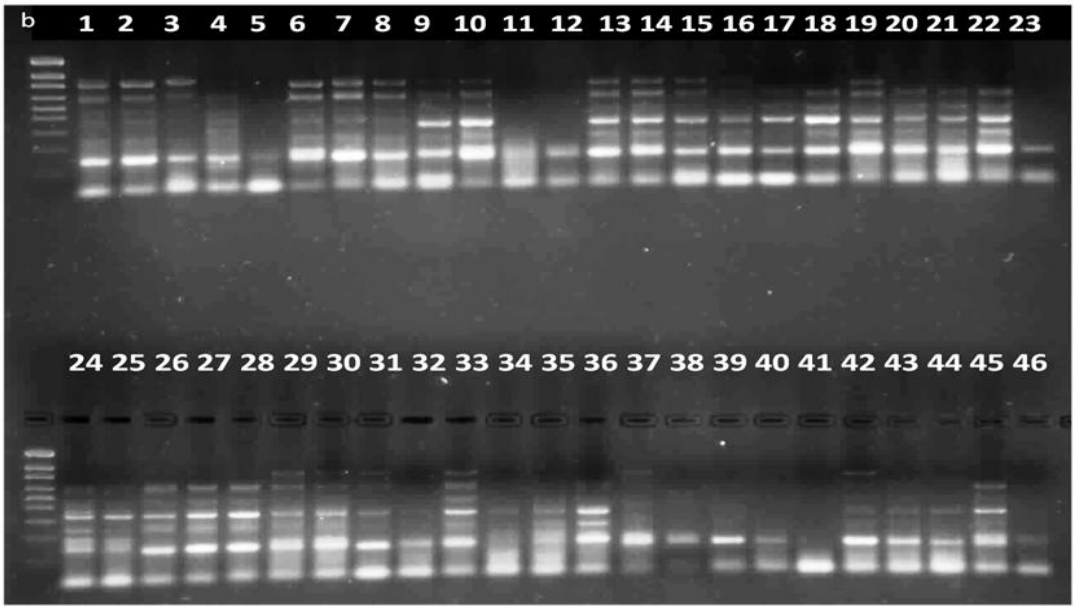

Figure 4.6: ISSR gel profile of primer UBC-808. The numbers from 1-46 are showing the Citron accessions used for the study

Correlation value (r) was calculated as 0.99 by Mantel test, which showed a very good fit of the clustering pattern to the data. A 2D plot generated from principle co-ordinate analysis (PCoA) of ISSR data also supported the clustering pattern of the UPGMA dendrogram (Figure 4.8). First and second principal components accounted for 23.48 and 15.44 %, respectively, of the total variation. Other genetic diversity parameters, such as observed number of alleles (na), effective number of alleles (ne), total heterozygosity (Ht), and Shannon's information index (I) were recorded as 1.981, 1.519, 0.312 and 0.475, respectively.

Table 4.5: Details of amplified bands generated by 11 ISSR primers in 46 accessions of *C. medica*

Sl. No.	Primer code	Sequence (5'-3')	Total no. of bands	PB[a]	PB[b]	PIC[c]	Amplified Fragment Length
1.	UBC-808	(AG)8C	14	11	79	0.4069	310-565
2.	UBC-809	(AG)8G	10	8	80	0.4021	280-982
3.	UBC-810	(GA)8T	12	9	75	0.1398	300-746
4.	UBC-811	(GA)8C	11	8	73	0.3781	250-824
5.	UBC-812	(GA)8A	10	7	70	0.2632	275-750
6.	UBC-834	(AG)8YT	11	8	73	0.3395	332-735
7.	UBC-836	(AG)8YA	9	7	78	0.3503	360-1350
8.	UBC-842	(GA)8YG	8	6	75	0.3641	140-325
9.	UBC-868	(GAA)6	7	5	72	0.3215	385-642
10.	UBC-880	(GGGTG)3	5	3	60	0.3829	745-962
11.	UBC-889	DBD(AC)7	8	5	63	0.3081	298-805
		Total	105	77	73 %	0.3324	

aTotal Polymorphic Bands, **b** Percentage of Polymorphic Bands, **c** Polymorphic Information Content (Average)

Table 4.6: Unique bands for ISSRmarkers identified in the present study

Sl.No	Primers	Number of bands	Accession no	Band size
1.	UBC-808	1	MS-42	310
2.	UBC-809	1	MD-11/65	700
3.	UBC-810	1	MD-22	440
4.	UBC-834	1	MD-22	350
	Total	4		

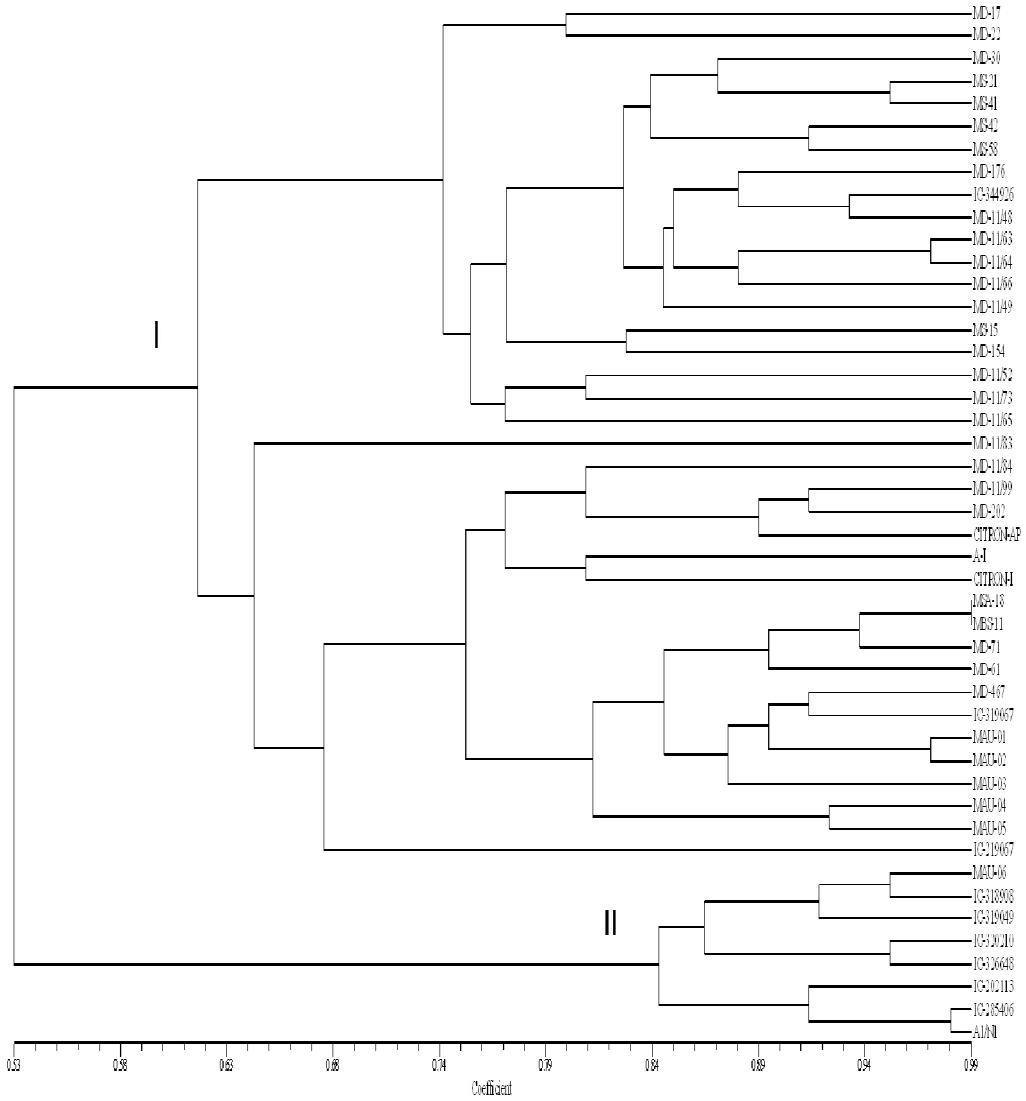

Figure 4.7: UPGMA dendrogram of 46 Indian citron accessions based on ISSR marker

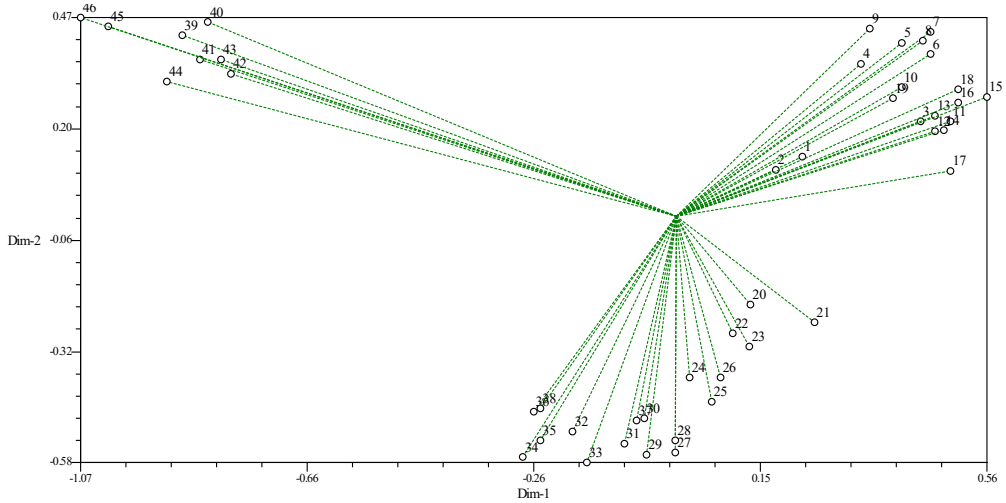

Figure 4.8: 2D plot of 46 Indian citron accessions based on ISSR data

6.2.3 SSR analysis

After screening 40 primers, 16 primers producing polymorphic and repeatable products were used for further analysis. The banding profile and polymorphism generated using the primers CAG-01 (Figure 4.9) is shown. PCR amplification of the DNA isolated from 46 citron accessions yielded a total of 66 alleles, of which 52 alleles (78.79%) were polymorphic. The total number of alleles produced varied from 3 to 7 alleles per locus with an average of 4.2 alleles per locus. The number of polymorphic amplicons for each primer ranged from 2 to 6 (average 3.3) loci per primer. PIC values were detected for individual primer and ranged from 0.18 to 0.42 with an average of 0.30. The maximum number of polymorphic alleles (7) was obtained with primer UCM-20 and the minimum number (2) was obtained with primer CAG-01 and CT-02. Overall size of the PCR amplified products ranged from 120-720bp (Table 4.7).

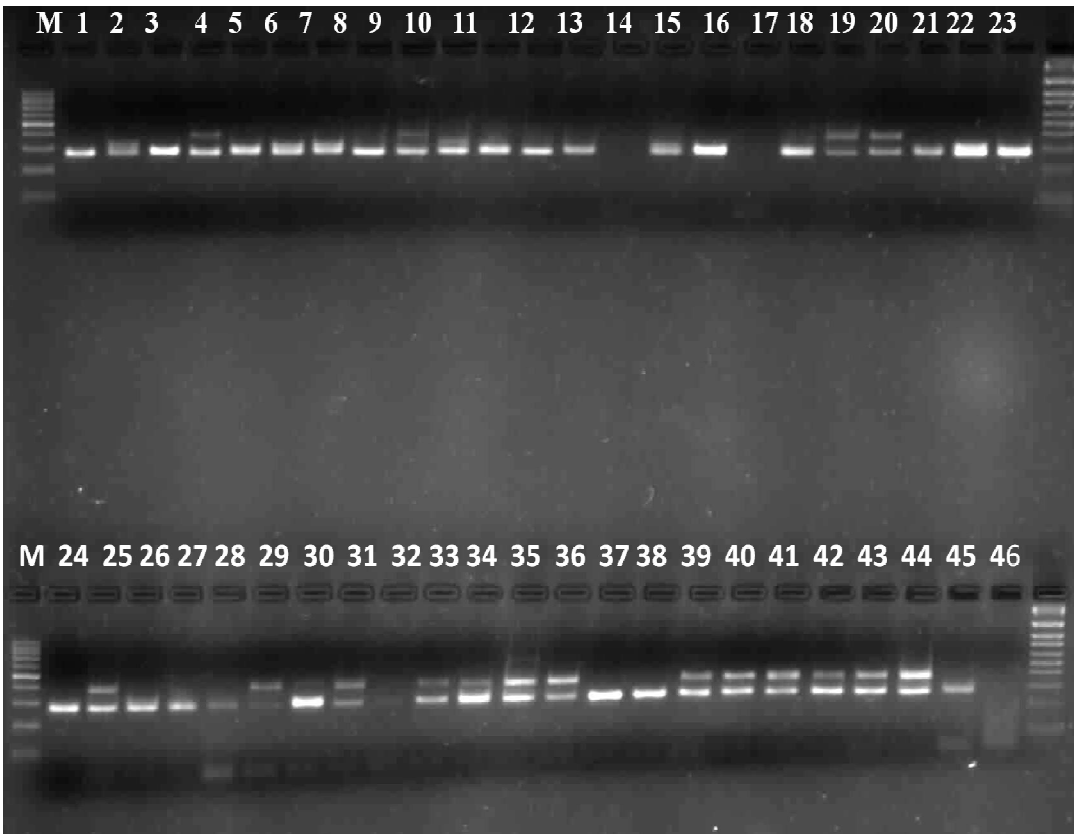

Figure 4.9: A representative SSR gel profile given by CAG 01 primer. The numbers from 1-46 are showing the Citron accessions used for the study

The SSR primers also allowed the detection of specific alleles present in some accessions and absent in others (Table 4.8). These primers produced a specific DNA allele which distinguished one accessions from the rest. Each of the primer CAG-01, UCM-05 and UCM-20 generated unique alleles in accession MD-17. These primers could be called "accession specific primers". Similarresults were detected with the primer CT-02 in MAU-01andIPS/PNB/319, UCM-08 in MSA-11/18 and UCM-17 in MD-22accessions, respectively.

The genetic similarity matrices between the *C. medica*accessions were determined on the basis of Nei's pairwise method.A maximum similarity value of 0.97 was observed between accession IC-326648and IC-320210, whereas MD-17 (Bemberia) and MAU-01 showed the similarity coefficient value of 0.42 indicating their distinctness. Average similarity across all accessions was about 0.70.

The UPGMA dendrogram based on Jaccard coefficient segregated the 46 *C. medica* accessions into two main clusters (Figure 4.10). The two clusters were further divided into various subgroups according to similarity between them. The two main clusters branched at similarity value of 0.64.Cluster I consisted of the larger clusters (44 accessions) which was further divided into 2 sub-clusters. The accessions IC-326648and IC-320210 were found to be closely related to each other with a similarity value of 0.97. In Cluster I, MD-17 accession was found to be distinctly related to other accessions.

The second main cluster consisted of only two accessions viz. MD-11/73 and MD-11/83 which clustered together with a similarity value of 0.89

A 2D plot generated from PCA of SSR data also supported the clustering pattern of UPGMA dendrogram (Figure 4.11). The first ten PCs contributed 65.89 % of the total variability of the analyzed accessions. The first five PCs accounted for 43.79 % of the total variability and the first three accounted for 30.35 % of the variance, in which maximum variability was contributed by first component (13.08 %) followed by second component and third component (8.89 and 8.38 %, respectively).

Table 4.7: Details of amplified bands generated by 16 SSR primers in 46 accessions of *C. medica*

Sl. no.	SSR loci	Repeat motiff	Forward primer	Reverse primer	Alleles	PB	PIC	Size range
1.	AC01	CA/TA	TTTGACATCAACATAAAACAAGAAA	TTTTAAAATCCCTGACCAGA	4	4	0.2890	160-195
2	AG14	GA	AAAGGGAAAGCCCTAATCTCA	CTTCCTCTTGCGGAGTGTTC	4	4	0.2334	130-170
3.	ATC09	TCA	TTCCTTATGTAATTGCTCTTTG	TGTGAGTGTTTGTGCGTGTG	4	3	0.3342	305-340
4.	CAG01	AGC	AACACTCGCACCAAATCCTC	TAAATGGCAACCCCAGCTTTG	3	2	0.3359	120-155
5.	CAT01	CAT/CTT	GCTTTCGATCCCTCCACATA	GATCCCTACAATCCTTGGTCC	5	3	0.2459	130-170
6.	CCT01	CCT	TCAACACCTCGAACAGAAGG	CCCACATGCTAGCACAAAGA	4	3	0.3345	150-195
7.	CT02	CT	ACGGTGCGTTTTGAGGTAAG	TGACTGTTGGATTTGGGATG	4	2	0.3558	145-180
8.	CT19	TC	CGCCAAGCTTACCACTCACTAC	GCCACGATTTGTAGGGGATAG	4	3	0.2304	140-170
9.	CT21	TC	CGAACTCATTAAAAGCCGAAAC	CAACAACCACCACTCTCACG	4	3	0.3691	135-175
10.	CTT01	CTT	TCAGACATTGAGTTGCTCG	TAACCACTTAGGCTTCGGCA	3	3	0.2916	680-720
11.	GT03	GT	GCCTTCTTGATTTACCGGAC	TGCTCCGAACTTCATCATTG	4	4	0.4216	275-325
12.	UCM05	CT	GTGCTCTCTCTGTTGACTCTCT	ACTTCTGCTATTACTTCTTCCG	4	3	0.3247	230-280
13.	UCM06	TC/GT	TTCTCTCTCTTCTCTCTCTCCA	AGTTGTTGTCATCGTCATAGC	3	3	0.2316	260-300
14.	UCM08	TA/CA	AGATCCAGTTAAAACACTCCAC	GTTAGTAGAAGATCCCAAAACG	4	3	0.3049	320-360
15.	UCM17	CTC	CTCGCTCTTCACTAGCATAGAT	CTCCTTCCTCTTAGCCTTTATT	5	3	0.1797	280-350
16.	UCM20	GT/GC	GGGTCTTGTTCTTGTAGTGATT	GGATAGAGGGCTTGTTATTGTA	7	6	0.2466	175-345
					66	52	0.2965	

PB- Polymorphic band, PIC- Polymorphic Information Content

Table 4.8: Unique bands for SSR markers identified in the present study

Sl. No	Primers	Number of bands	Band size (bp)	Accession no
1.	CAG-01	1	130	MD-17
2.	CT-02	1	180	MAU-01
		1	150	IPS/PNB/319
3.	UCM-05	1	240	MD-17
4.	UCM-08	1	320	MSA-11/18
5.	UCM-17	1	320	MD-22
6.	UCM-20	1	320	MD-17

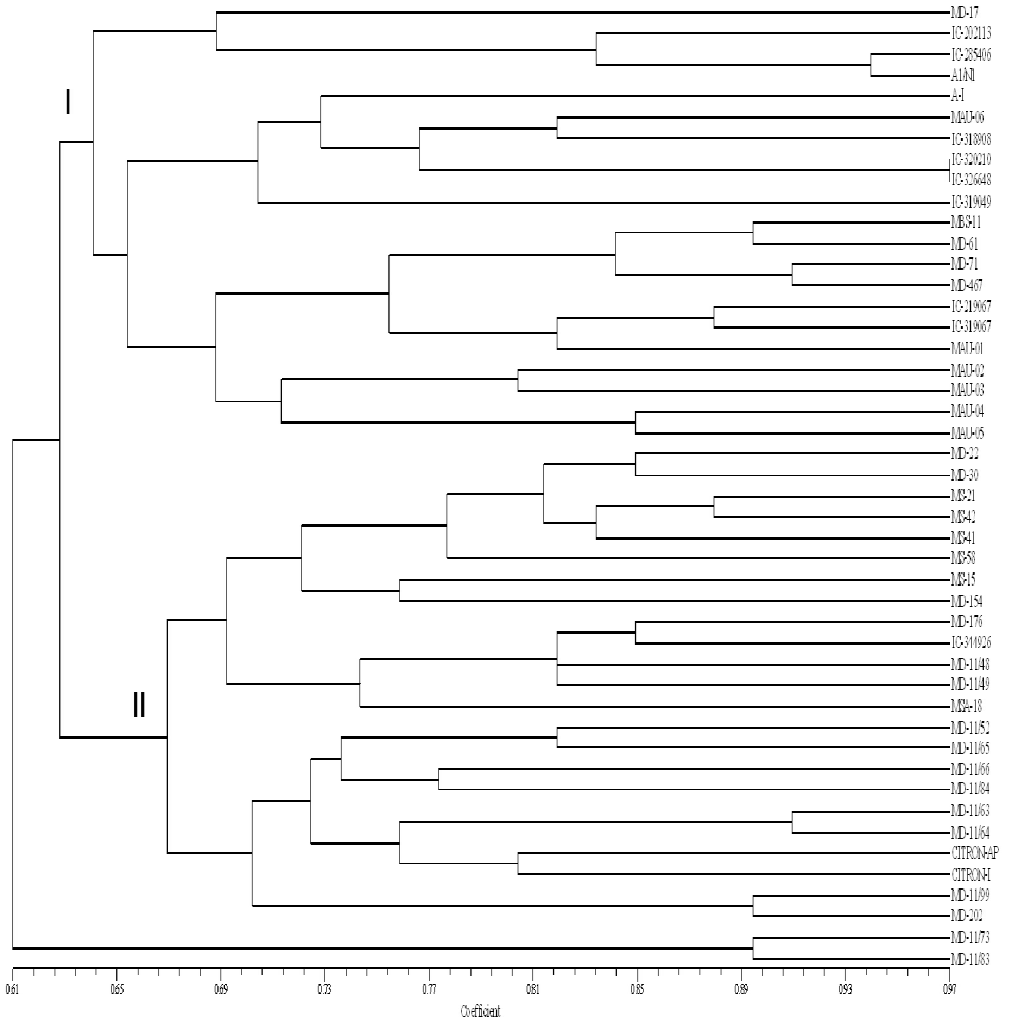

Figure 4.10: UPGMA dendrogram of 46 *C. medica* accessions based on 16 SSR markers

The performance of microsatellite marker was evaluated using various parameters such as percentage of polymorphism, average bands and polymorphic bands per primer, polymorphic information content (PIC), average similarity values, observed number of alleles (na), effective number of alleles (ne), total heterozygosity (Ht), Shannon's information index (I). The values of genetic structure data like observed number of alleles (na), effective number of alleles (ne), total heterozygosity (Ht) and Shannon's information index (I) were recorded to be 2.000, 1.429, 0.266 and 0.417, respectively.

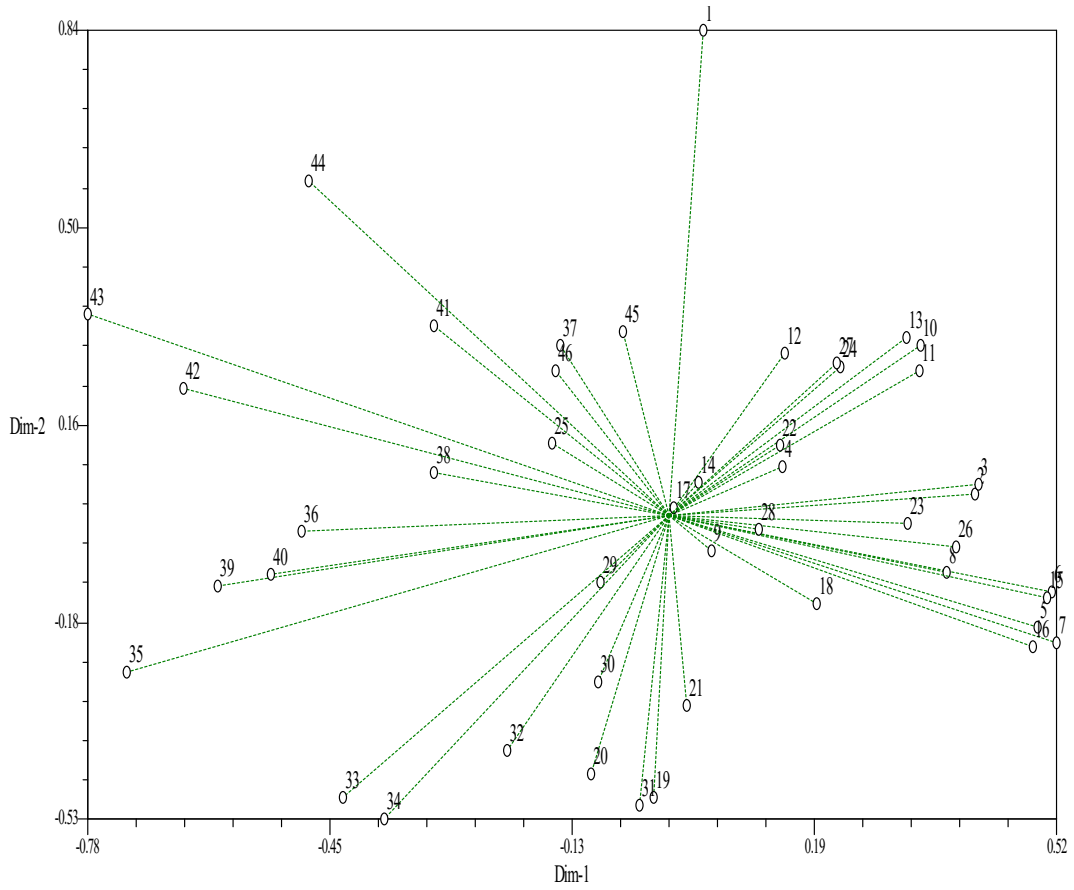

Figure 4.11: 2D plot of 46 Indian citron accessions based on 16 SSR markers

6.2.4 Cumulative analysis of RAPD, ISSR and SSR data

The binary data of 46 accessions of *C. medica*, derived from RAPD, ISSR and SSR analysis were pooled to form a single data set. Similarity value ranged from 0.49 to 0.96 with an average of 0.72. Similarity value was found minimum (0.49) between IC-326648 and MD-11/64; IC-202113 and MD-11/65and maximum (0.96) between A1N1 and IC-285406 accessions. The UPGMA-based dendrogram from the combined data set separated all the accessions in two main clusters (Figure 4.12). Cluster I contained 23 accessions represented from North-Eastern states i.e., Assam, Meghalaya, Nagaland and Sikkim. Accession MD-17 (Bemberia) and MD-22 (Bemberia) formed a separate sub-cluster within the main cluster. The second cluster also comprised 23 accessions and represented accessions from Arunachal Pradesh, Punjab, Himachal Pradesh, Uttarakhand and Uttar Pradesh. In Cluster II, Tayum cultivars (Citron I, A-I and Citron-AP) from

Arunachal Pradesh was found to be distinct as it grouped into a separate sub-cluster within the main cluster.

A 2D plot generated from PCA of RAPD, ISSR and SSR data (Figure 4.13) also supported the clustering pattern of UPGMA dendrogram. The first ten PCs contributed 61.81% of the total variability of the analyzed accessions. The first five PCs accounted for 43.96% of the total variability and the first three accounted for 34.17% of the variance, in which maximum variability was contributed by first component (20.21%) followed by second component and third component (8.35 and 5.62 %, respectively).A combined data of SSR, ISSR and RAPD similarity matrices were used for co-phenetic analysis, which showed very significant correlation (r = 0.96) among the three separate data sets.

The values of genetic structure data like observed number of alleles (na), effective number of alleles (ne), total heterozygosity (Ht) and Shannon's information index (I) were recorded to be 1.9905, 1.455, 0.278 and 0.431, respectively.

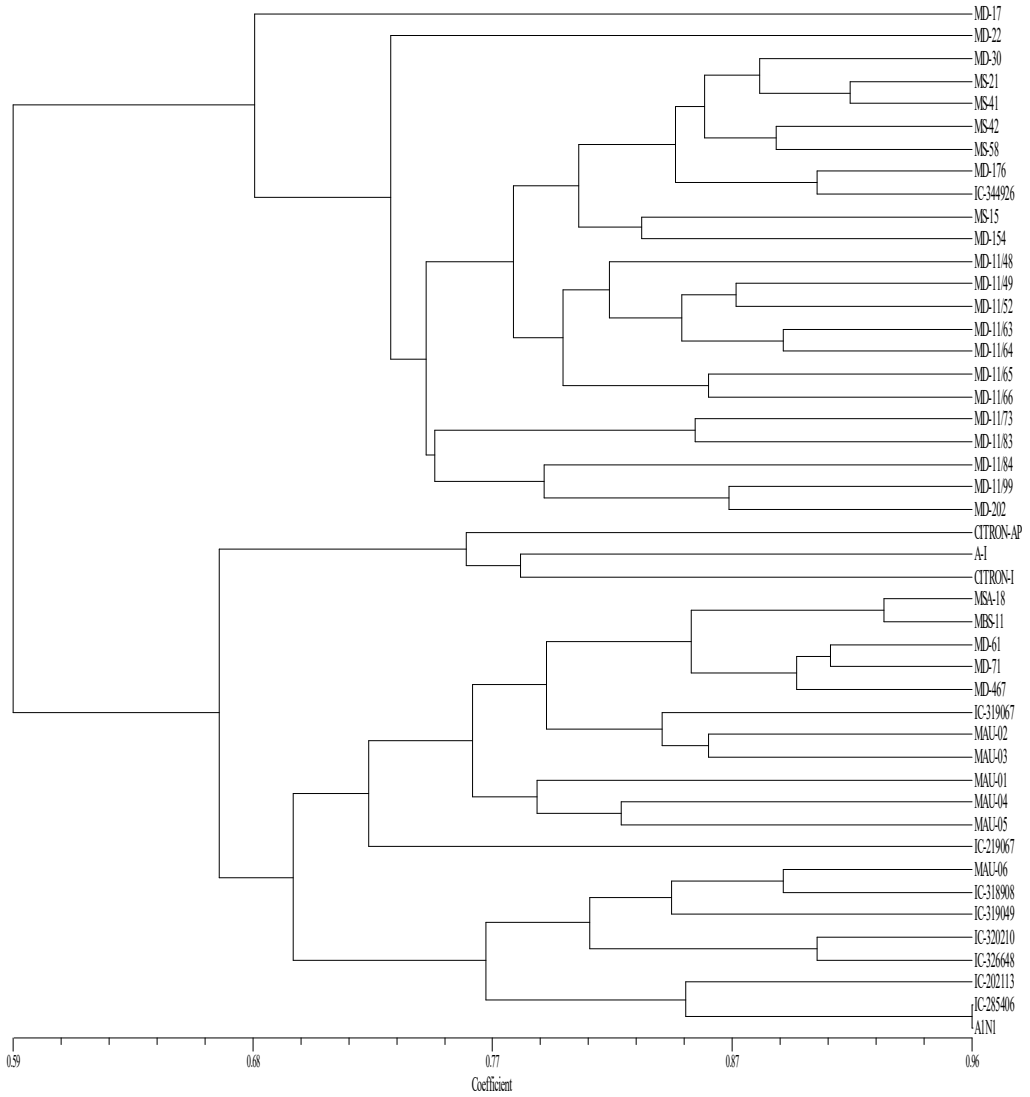

Figure 4.12: UPGMA dendrogram of 46 Indian citron accessions based on combined data of RAPD, ISSR and SSR markers

The performance of two markers was evaluated using various parameters such as percentage of polymorphism, average bands and polymorphic bands per primer, polymorphic information content (PIC), average similarity values, observed number of alleles (na), effective number of alleles (ne), total heterozygosity (Ht), Shannon's information index (I) (Table 4.9).

Table 4.9: Comparison of polymorphism and population genetic data generated by RAPD, ISSR and SSR primers in 46 accessions of *C. medica*

Marker system (Total primers used)	PPB	Avg no. of bands/ Primer	Avg no. of PB/ primer	Mean PIC	Range of similarity value (Avg.)	na	ne	I	Ht
RAPD (17)	82	12.5	10	0.2965	0.73	1.9906	1.4180	0.2567	0.4001
ISSR (11)	73	9.5	7	0.3324	0.68	1.9810	1.5178	0.3116	0.4742
SSR (16)	78.79	4.2	3.3	0.2965	0.69	2.000	1.4297	0.2664	0.4174
Combined (44)	77.93	8.73	6.77	0.3084	0.70	1.9905	1.4552	0.2782	0.4306

(PPB- Percentage of Polymorphic Bands; PB- Polymorphic Bands; PIC- Polymorphic Information Content; MI- Marker Index; na - Observed number of alleles ; ne - Effective number of alleles; I - Shannon's information index; Ht- Total heterozygosity)

Table 4.10: Eigen values, differences, percentage of proportions and cumulative for 10 principal co-ordinate axes derived from combined RAPD, ISSR and SSR data of *C. medica*

Sl. No.	Eigenvalue	Percent	Cumulative
1	9.09206	20.2046	20.2046
2	3.757126	8.3492	28.5537
3	2.527563	5.6168	34.1706
4	2.267838	5.0396	39.2102
5	2.135718	4.746	43.9562
6	1.77986	3.9552	47.9115
7	1.717658	3.817	51.7285
8	1.597849	3.5508	55.2793
9	1.556673	3.4593	58.7385
10	1.378521	3.0634	61.8019

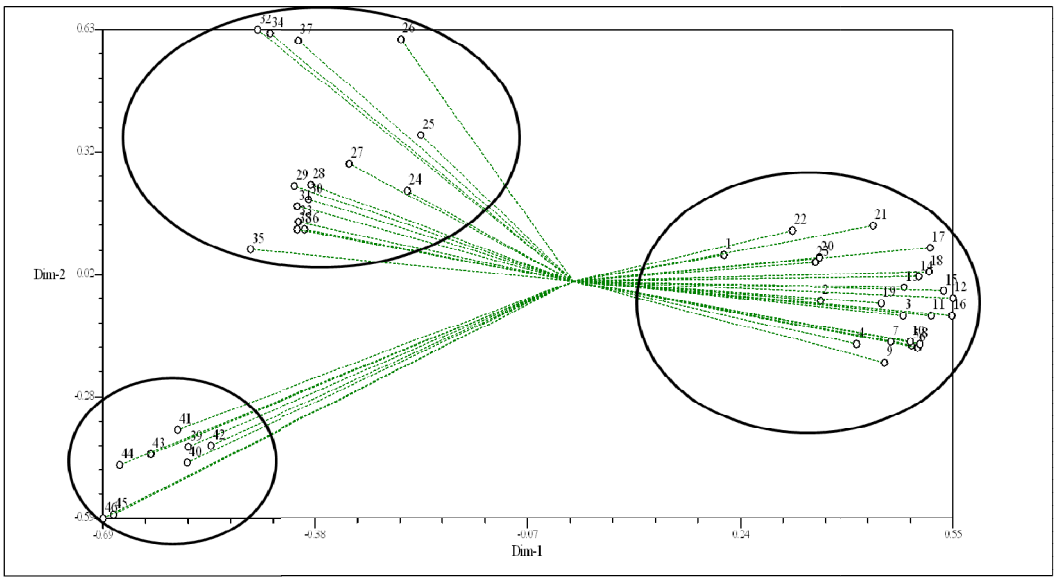

Figure 4.13: 2-D Plot of 46 Indian citron accessions based on combined data of RAPD, ISSR and SSR

6.3 Phylogenetic relationship analysis

6.3.1 Sequence analysis of *matK* gene

The BLASTn search helped determine that the new sequences were from sequence region and maximum homology was obtained from the sequences of *Citrus*. Sequence length of *matK* in the 23 *Citrus* accessions ranged from 736 to 862bp (avg. Sequence length 824bp). The data set including alignment gaps and missing data comprised 887bp aligned nucleotide positions, which included 786 conserved sites, 100 variable sites and 65 parsimony informative sites. In the *matK* sequences, G+C content ranged from 32.9% to 35.2% with an average of 34.2%. Transition/Transversion bias (R) is 0.40. Substitution pattern and rates were estimated under the Kimura (1980) 2-parameter model (+G+I). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+G], parameter = 0.6399). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 65.4397% sites). The nucleotide frequencies are A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%. All positions containing gaps and missing data were eliminated. There were a total of 726 positions in the final dataset.Summary of *matK* sequence data is given in Table 4.11. The *matK* sequence analysis showed moderate rate of nucleotide divergence within the *Citrus* taxa. Genetic divergence within *Citrus* group ranged from 0 (among citron accessions) to 0.021 (*C. ichangensis* and *C. jambhiri)* with an average of 0.007.

The phylogeny among *Citrus* genotypes was constructed through NJ method. In the NJ bootstrap consensus tree (Figure 4.14), all the *Citrus* accessions were grouped into five distinct clusters:

Cluster I: *C. ichangensis*, *C. maxima* and *C. latipes*

Cluster II: *C. sinensis*, *C. karna* and *C. limettioides*

Cluster III: *C. macroptera*, *C. aurantifolia* and *C. limon*

Cluster IV: *C. reticulata*, *C. jambhiri*, *C. aurantium* and *C. limonia*

Cluster V: *C. medica* and *C. indica*

Table 4.11: Summary of cpDNA*matK* sequences of 23 accessions of *Citrus* and the outgroup, *Poncirus trifoliata*

sl no.	Species	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos #1	T-2	C-2	A-2	G-2	Pos #2	T-3	C-3	A-3	G-3	Pos #3
1	*C.medica*-AP	32.0	14.8	34.9	18.3	765.0	32	12.2	37.3	18.8	255.0	33	13.7	37.3	16.5	255.0	32	18.4	30.2	19.6	255.0
2	*C.medica* MD-66	32.1	15.5	34.1	18.3	826.0	31	13.4	37.3	18.1	276.0	32	15.2	36.1	17.0	277.0	33	17.9	28.9	19.8	273.0
3	*C.medica* MD-99	32.0	15.5	34.2	18.3	825.0	31	13.1	37.8	18.2	275.0	32	15.2	35.7	17.0	277.0	33	18.3	28.9	19.8	273.0
4	*C.medica* MSA-18	32.1	15.5	34.1	18.3	825.0	31	13.1	37.8	18.2	275.0	32	15.2	35.5	17.0	276.0	33	18.2	28.8	19.7	274.0
5	*C.medica* Sikkim	32.3	14.3	34.8	18.6	753.0	32	11.6	37.1	19.1	251.0	33	13.9	36.9	16.7	252.0	32	17.6	30.4	20.0	250.0
6	Themachhi MS-58	32.3	14.5	34.5	18.6	736.0	32	11.8	36.7	19.2	245.0	33	14.2	36.2	17.1	246.0	32	17.6	30.6	19.6	245.0
7	*C.limon* AL	31.8	14.6	34.8	18.8	752.0	31	12.0	37.8	19.3	249.0	33	13.9	36.5	16.7	252.0	31	17.9	30.3	20.3	251.0
8	*C.aurantifolia*	32.1	15.4	33.6	18.9	853.0	31	12.7	37.1	18.7	283.0	33	14.0	35.8	16.8	285.0	32	19.3	28.1	21.1	285.0
9	*C.limettioides*	32.7	16.8	32.1	18.4	853.0	32	14.1	35.3	18.7	283.0	33	15.5	34.2	16.9	284.0	33	20.6	26.9	19.6	286.0
10	*C.limonia*	32.6	16.5	32.3	18.6	854.0	31	14.8	35.6	18.7	284.0	33	14.7	34.7	17.2	285.0	33	20.0	26.7	20.0	285.0
11	*C.jambhiri*	33.3	15.3	32.6	18.8	847.0	33	13.1	35.7	18.4	283.0	33	14.2	34.5	18.1	281.0	34	18.7	27.6	19.8	283.0
12	*C.karna*	32.3	15.8	33.1	18.8	847.0	31	13.5	36.3	18.9	281.0	33	14.8	35.0	17.7	283.0	33	19.1	27.9	19.8	283.0
13	*C.reticulata*	33.0	16.5	32.0	18.5	855.0	33	14.0	35.0	18.5	286.0	33	15.5	34.2	17.3	284.0	33	20.0	27.0	19.6	285.0
14	*C.maxima*	32.2	15.9	33.0	18.9	861.0	31	13.6	36.6	19.2	287.0	33	15.0	34.5	17.8	287.0	33	19.2	27.9	19.9	287.0
15	*C.sinensis*	32.6	14.8	34.0	18.5	755.0	33	12.3	36.5	18.7	252.0	33	14.3	35.7	17.1	252.0	32	17.9	29.9	19.9	251.0
16	*C.aurantium*	32.3	15.5	33.1	19.1	862.0	31	13.2	36.8	18.8	288.0	33	15.0	34.3	18.2	286.0	33	18.4	28.1	20.5	288.0
17	*C.indica* S-44	32.7	15.8	32.9	18.5	859.0	32	13.3	35.7	18.9	286.0	33	14.6	35.2	17.1	287.0	33	19.6	28.0	19.6	286.0
18	*C.indica* S-51	33.4	15.0	33.6	18.0	848.0	32	12.7	36.4	18.7	283.0	34	14.1	35.3	16.6	283.0	34	18.1	29.1	18.8	282.0
19	*C.latipes* S-01	33.4	15.6	32.7	18.3	851.0	32	12.8	36.9	18.1	282.0	34	15.1	34.0	17.2	285.0	34	19.0	27.1	19.7	284.0
20	*C.latipes* S-47	32.4	16.2	32.7	18.7	859.0	31	13.7	36.5	18.6	285.0	33	15.3	34.1	17.4	287.0	33	19.5	27.5	20.2	287.0
21	*C.macroptera* S-27	32.2	16.1	32.7	19.0	857.0	32	13.0	36.1	19.3	285.0	33	15.7	34.5	17.1	287.0	32	19.6	27.4	20.7	285.0
22	*C.macroptera* S-87	31.1	16.0	34.4	18.6	808.0	30	13.0	37.9	19.0	269.0	32	15.2	35.9	16.7	270.0	31	19.7	29.4	20.1	269.0
23	*C.ichangensis*	32.4	16.5	32.7	18.3	852.0	30	14.1	37.1	18.4	283.0	33	15.8	34.0	16.8	285.0	33	19.7	27.1	19.7	284.0
24	*Poncirus trifoliata*	31.5	15.1	34.6	18.8	777.0	31	12.0	38.0	19.4	258.0	32	13.8	36.9	17.3	260.0	32	19.3	29.0	19.7	259.0
	Average	32.4	15.6	33.4	18.6	824.2	32	13.1	36.7	18.7	274.3	33	14.8	35.3	17.1	275.3	33	18.9	28.4	19.9	274.6

P. trifoliata was separately attached at the base of the tree as the diverging *Citrus* relative's lineage. The phylogeny was also inferred through the maximum parsimony method which separated all the 23 accessions into five clusters as similar to NJ tree (Figure 4.15).

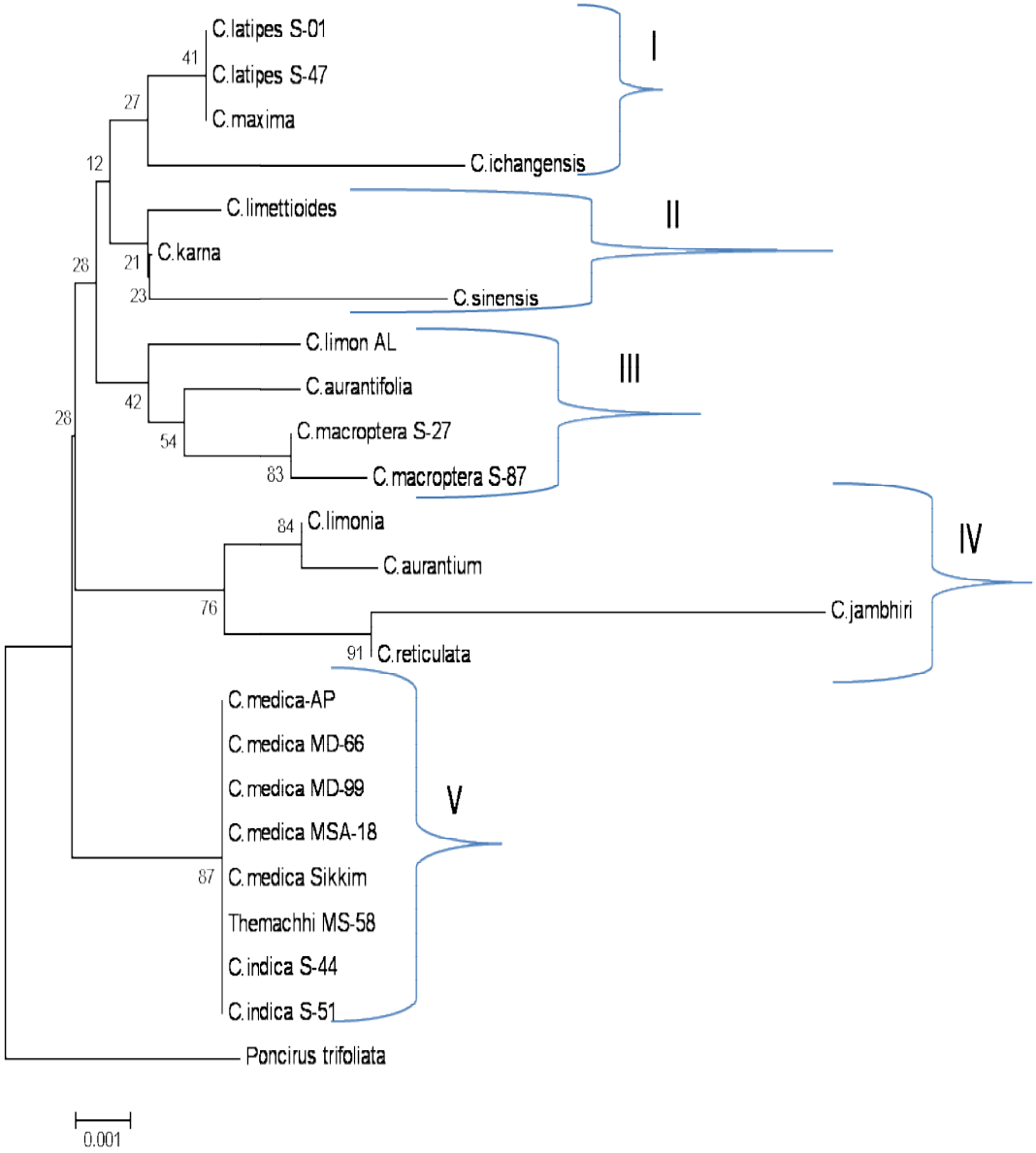

Figure 4.14: NJ bootstrap consensus tree of 23 accessions of *Citrus* and the out-group, *Poncirus trifoliata* from *matK* sequence data analysis. Numbers are bootstrap values based on 500 re-sampling

Table 4.12: Pairwise genetic divergence of 23 *Citrus accessions* and the outgroup *Poncirus trifoliata* based on *matK* analysis

***C.medica*-AP**																								
***C.medica*_MD-66**	0.000																							
***C.medica*_MD-99**	0.000	0.000																						
***C.medica*_MSA-18**	0.000	0.000	0.000																					
***C.medica*_Sikkim**	0.000	0.000	0.000	0.000																				
Themachhi_MS-58	0.000	0.000	0.000	0.000	0.000																			
***C.limon*_AL**	0.007	0.007	0.007	0.007	0.007	0.007																		
C.aurantifolia	0.007	0.007	0.007	0.007	0.007	0.007	0.006																	
C.limettioides	0.006	0.006	0.006	0.006	0.006	0.006	0.004	0.007																
C.limonia	0.007	0.007	0.007	0.007	0.007	0.007	0.008	0.008	0.007															
C.jambhiri	0.017	0.017	0.017	0.017	0.017	0.017	0.018	0.018	0.017	0.013														
C.karna	0.004	0.004	0.004	0.004	0.004	0.004	0.006	0.006	0.001	0.006	0.015													
C.reticulata	0.008	0.008	0.008	0.008	0.008	0.008	0.010	0.010	0.008	0.004	0.008	0.007												
C.maxima	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.006	0.003	0.007	0.017	0.001	0.008											
C.sinensis	0.010	0.010	0.010	0.010	0.010	0.010	0.011	0.011	0.007	0.011	0.018	0.006	0.010	0.007										
C.aurantium	0.008	0.008	0.008	0.008	0.008	0.008	0.010	0.010	0.008	0.001	0.014	0.007	0.006	0.008	0.013									
***C.indica*_S-44**	0.000	0.000	0.000	0.000	0.000	0.000	0.007	0.007	0.006	0.007	0.017	0.004	0.008	0.006	0.010	0.008								
***C.indica*_S-51**	0.000	0.000	0.000	0.000	0.000	0.000	0.007	0.007	0.006	0.007	0.017	0.004	0.008	0.006	0.010	0.008	0.000							
***C.latipes*_S-01**	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.006	0.003	0.007	0.017	0.001	0.008	0.000	0.007	0.008	0.006	0.006						
***C.latipes*_S-47**	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.006	0.003	0.007	0.017	0.001	0.008	0.000	0.007	0.008	0.006	0.006	0.000					
***C.macroptera*_S-27**	0.007	0.007	0.007	0.007	0.007	0.007	0.006	0.004	0.007	0.008	0.018	0.006	0.010	0.004	0.011	0.010	0.007	0.007	0.004	0.004				
***C.macroptera*_S-87**	0.008	0.008	0.008	0.008	0.008	0.008	0.007	0.006	0.008	0.010	0.020	0.007	0.011	0.006	0.013	0.011	0.008	0.008	0.006	0.006	0.001			
C.ichangensis	0.010	0.010	0.010	0.010	0.010	0.010	0.011	0.010	0.010	0.011	0.021	0.008	0.013	0.007	0.014	0.013	0.010	0.010	0.007	0.007	0.008	0.010		
Poncirus_trifoliata	0.008	0.008	0.008	0.008	0.008	0.008	0.010	0.010	0.008	0.010	0.020	0.007	0.011	0.008	0.013	0.011	0.008	0.008	0.008	0.008	0.010	0.011	0.013	

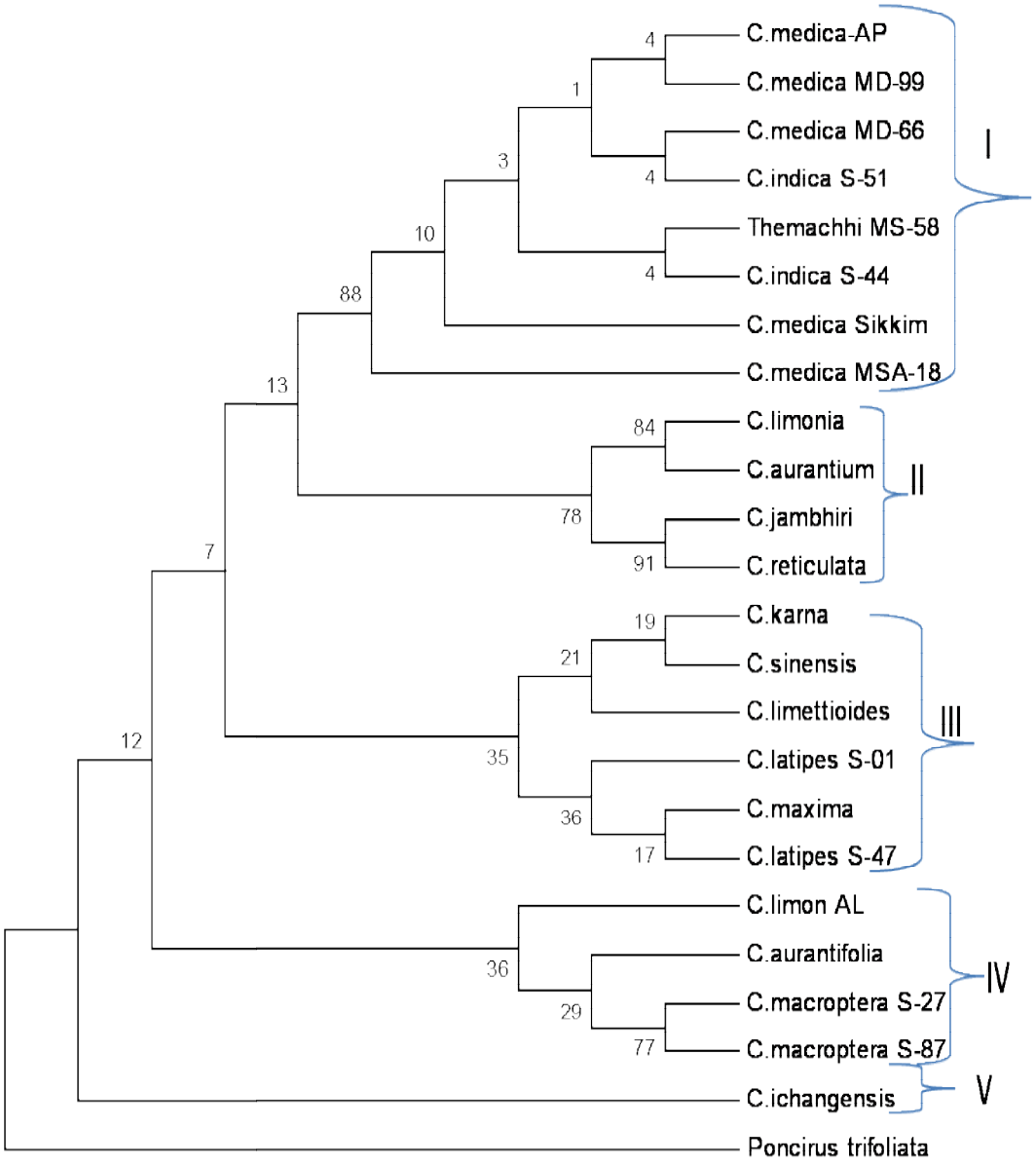

Figure 4.15: MP bootstrap consensus tree of 23 accessions of *Citrus* and the out-group, *Poncirus trifoliata* from *matK* sequence data analysis. Numbers are bootstrap values based on 500 re-sampling

6.3.2 Sequence analysis of *rbcL* gene

Sequence length of *rbcL* in the 23 *Citrus* accessions ranged from 1207 to 1297 bp (avg. sequence length 1245bp). The data set including alignment gaps and missing data comprised 1307bp aligned nucleotide positions, which included 1186 conserved sites, 115 variable sites and 56 parsimony informative sites. In the *rbcL* sequences, G+C

content ranged from 45.0% to 45.8% with an average of 45.4%. The estimated Transition/Transversion bias (R) is 0.82. Substitution pattern and rates were estimated under the Kimura (1980) 2-parameter model (+G+I). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+G], parameter = 0.1230). The nucleotide frequencies are A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%. All positions containing gaps and missing data were eliminated. There were a total of 1200 positions in the final dataset.Summary of *rbcL* sequence data is given in Table 4.13. The *rbcL* sequence analysis showed moderate rate of nucleotide divergence within the *Citrus* taxa. Genetic divergence within *Citrus* group ranged from 0.00 (among the citron accessions) to 0.034 (*C. karna, C. medica* -MD99, *C. karna, C. indica*) (avg. 0.017).

The phylogeny constructed based on NJ bootstrap consensus tree divided all the 23 Citrus accessions into five distinct clusters as shown in Figure 4.16.

Cluster I: *C. macroptera, C. sinensis, C. jambhiri, C. maxima, C. reticulata* and *C. karna*

Cluster II: *C. indica*

Cluster III: *C. medica*

Cluster IV: *C. limonia, C. limon* and *C. aurantifolia*

Cluster V: *C. latipes, C. ichangensis, C. limettioides* and *C. aurantium*

In *rbcL* sequence analysis also, *P. trifoliata* was found to be separately attached at the base of the tree as the diverging *Citrus* relative's lineage. The phylogeny inferred through the maximum parsimony method also separated all the 23 accessions into five distinct clusters as similar to NJ tree (Figure 4.17).

Table 4.13: Summary of cpDNA*rbc*L sequences of 23 accessions of *Citrus* and the outgroup, *Poncirus trifoliata*

Sl No.	Species name	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos #1	T-2	C-2	A-2	G-2	Pos #2	T-3	C-3	A-3	G-3	Pos #3
1	*C.medica* AP	28.3	20.0	26.5	25.3	1255.0	18	20.3	23.0	38.5	418.0	27	22.9	29.4	20.5	419.0	39	16.7	27.0	16.7	418.0
2	*C.medica* MD-66	28.1	20.0	26.5	25.3	1252.0	18	20.6	23.0	38.4	417.0	27	22.7	29.4	20.6	418.0	39	16.8	27.1	17.0	417.0
3	*C.medica* MD-99	28.2	20.0	26.5	25.2	1232.0	18	20.4	22.9	38.7	411.0	27	23.4	29.0	20.7	411.0	40	16.3	27.8	16.3	410.0
4	*C.medica* MSA-18	28.2	19.9	26.7	25.3	1242.0	18	20.3	23.2	38.4	414.0	27	22.9	29.4	20.5	415.0	39	16.5	27.4	16.9	413.0
5	*C.medica* Sikkim	28.2	20.0	26.3	25.5	1260.0	18	20.3	22.9	38.7	419.0	27	22.8	29.2	21.1	421.0	40	16.9	26.9	16.7	420.0
6	Themachhi MS-58	28.4	20.0	26.4	25.2	1259.0	18	20.3	22.9	38.7	419.0	27	23.1	29.3	20.2	420.0	40	16.7	27.1	16.7	420.0
7	*C.limon* AL	28.4	20.2	26.3	25.1	1248.0	19	20.2	22.4	38.6	415.0	27	23.0	29.5	20.4	417.0	39	17.3	26.9	16.3	416.0
8	*C.aurantifolia*	28.5	19.9	26.3	25.3	1250.0	19	20.0	22.6	38.9	416.0	27	22.8	29.5	20.4	417.0	40	17.0	26.9	16.5	417.0
9	*C.limettioides*	28.5	20.1	26.4	25.0	1209.0	19	20.3	23.1	37.7	403.0	27	23.1	29.8	20.6	403.0	40	16.9	26.3	16.6	403.0
10	*C.limonia*	28.3	20.6	25.9	25.2	1297.0	18	21.1	22.2	38.4	432.0	27	23.1	29.2	20.4	432.0	39	17.6	26.3	16.9	433.0
11	*C.jambhiri*	28.4	20.2	26.0	25.4	1232.0	18	20.4	22.6	38.4	411.0	27	23.4	29.0	20.7	411.0	40	16.8	26.3	17.1	410.0
12	*C.karna*	28.5	20.6	26.0	24.9	1207.0	19	21.4	22.6	37.3	402.0	27	23.1	29.4	20.1	402.0	39	17.4	26.1	17.1	403.0
13	*C.reticulata*	28.6	20.4	25.9	25.1	1225.0	19	20.3	22.3	38.0	408.0	27	23.8	28.7	20.6	408.0	39	17.1	26.7	16.9	409.0
14	*C.maxima*	28.8	20.3	26.1	24.8	1212.0	19	20.3	23.0	37.4	404.0	28	23.3	29.0	20.0	404.0	39	17.3	26.2	17.1	404.0
15	*C.sinensis*	28.5	20.4	25.8	25.3	1211.0	19	20.5	22.5	37.9	404.0	27	23.3	29.3	20.3	403.0	39	17.3	25.7	17.6	404.0
16	*C.aurantium*.AB505953	28.1	19.9	26.6	25.3	1295.0	18	20.2	23.7	38.3	431.0	27	22.6	29.6	21.0	433.0	40	16.9	26.7	16.7	431.0
17	*C.indica* S-44	28.4	19.9	26.6	25.1	1259.0	18	20.3	23.4	38.2	419.0	28	22.9	29.8	19.8	420.0	39	16.7	26.7	17.4	420.0
18	*C.indica* S-51	28.2	20.2	26.6	25.0	1258.0	18	20.5	23.2	38.2	419.0	28	22.9	29.5	19.8	420.0	39	17.2	27.0	17.2	419.0
19	*C.latipes* S-01	28.5	20.3	26.3	24.9	1260.0	19	20.5	23.2	37.7	419.0	27	23.3	29.5	20.2	421.0	40	17.1	26.2	16.9	420.0
20	*C.latipes* S-47	28.4	20.3	26.3	25.0	1260.0	18	20.5	22.9	38.2	419.0	27	23.3	29.5	20.2	421.0	40	17.1	26.4	16.7	420.0
21	*C.macroptera* S-27	28.5	20.4	25.8	25.2	1212.0	19	20.3	22.5	38.1	404.0	27	23.3	29.0	20.8	404.0	40	17.6	26.0	16.8	404.0
22	*C.macroptera* S-87	28.5	20.5	25.8	25.2	1212.0	19	20.5	22.5	38.1	404.0	27	23.3	29.0	20.8	404.0	40	17.6	26.0	16.8	404.0
23	*C.ichangensis*	28.3	20.1	26.7	24.9	1244.0	19	20.5	23.7	37.2	414.0	27	23.1	29.4	20.5	415.0	39	16.6	27.0	17.1	415.0
24	*P.trifoliata*.AB505932	28.0	20.0	26.5	25.4	1292.0	18	20.5	23.7	38.1	430.0	27	22.5	29.4	21.3	432.0	40	17.2	26.5	16.7	430.0
	Average	28.4	20.2	26.3	25.2	1245.1	18	20.4	22.9	38.2	414.7	27	23.1	29.3	20.5	415.5	39	17.0	26.6	16.9	415.0

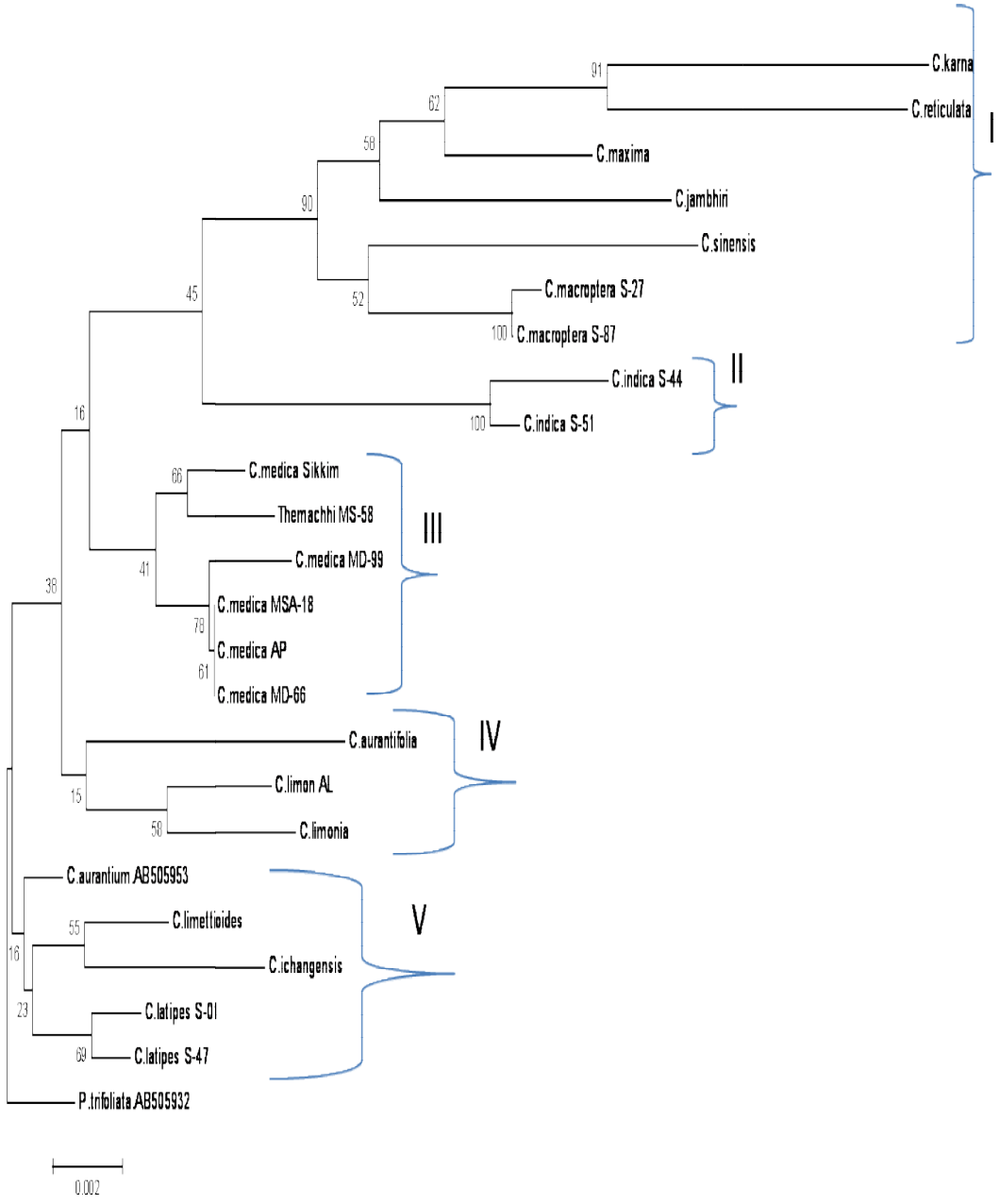

Figure 4.16: NJ bootstrap consensus tree of 23 accessions of *Citrus* and the out-group, *Poncirus trifoliata* from *rbcL* sequence data analysis. Numbers are bootstrap values based on 500 re-sampling

Table 4.14: Pairwise genetic divergence of 23 *Citrus* accessions and the outgroup *Poncirus trifoliata* based on *rbcL* analysis

***C. medica*_AP**																							
***C. medica*_MD-66**	0.000																						
***C. medica*_MD-99**	0.003	0.003																					
***C. medica*_MSA-18**	0.000	0.000	0.003																				
***C .medica*_Sikkim**	0.004	0.004	0.007	0.004																			
Themachhi_MS-58	0.005	0.005	0.008	0.005	0.004																		
C .limon_AL	0.010	0.010	0.012	0.010	0.014	0.013																	
C. aurantifolia	0.013	0.013	0.014	0.013	0.015	0.014	0.010																
C .limettioides	0.010	0.010	0.012	0.010	0.013	0.015	0.011	0.014															
C .limonia	0.011	0.011	0.013	0.011	0.015	0.016	0.007	0.016	0.010														
C .jambhiri	0.023	0.023	0.025	0.023	0.022	0.019	0.020	0.021	0.024	0.021													
C. karna	0.032	0.032	0.034	0.032	0.031	0.027	0.026	0.030	0.031	0.027	0.022												
C. reticulata	0.030	0.030	0.031	0.030	0.031	0.026	0.025	0.029	0.030	0.026	0.022	0.018											
C. maxima	0.023	0.023	0.025	0.023	0.022	0.019	0.016	0.021	0.020	0.018	0.016	0.016	0.019										
C. sinensis	0.026	0.026	0.028	0.026	0.022	0.023	0.021	0.025	0.022	0.023	0.022	0.024	0.029	0.018									
***C.aurantium*.AB505953**	0.007	0.007	0.008	0.007	0.011	0.012	0.008	0.011	0.003	0.007	0.020	0.027	0.026	0.017	0.022								
***C. indica*_S-44**	0.015	0.015	0.018	0.015	0.016	0.015	0.022	0.023	0.025	0.026	0.025	0.034	0.033	0.023	0.025	0.022							
***C. indica*_S-51**	0.013	0.013	0.015	0.013	0.013	0.013	0.019	0.020	0.023	0.024	0.024	0.031	0.031	0.020	0.022	0.019	0.004						
***C. latipes*_S-01**	0.009	0.009	0.011	0.009	0.012	0.014	0.010	0.014	0.008	0.009	0.019	0.028	0.027	0.014	0.017	0.005	0.019	0.017					
***C. latipes*_S-47**	0.009	0.009	0.011	0.009	0.012	0.014	0.012	0.013	0.006	0.011	0.021	0.028	0.027	0.016	0.018	0.004	0.019	0.017	0.003				
***C. macroptera*_S-27**	0.021	0.021	0.023	0.021	0.017	0.018	0.016	0.019	0.017	0.019	0.018	0.024	0.023	0.012	0.014	0.019	0.021	0.019	0.014	0.014			
***C. macroptera*_S-87**	0.020	0.020	0.022	0.020	0.016	0.017	0.015	0.019	0.016	0.018	0.017	0.023	0.024	0.011	0.013	0.018	0.020	0.018	0.013	0.013	0.001		
C .ichangensis	0.013	0.013	0.014	0.013	0.013	0.016	0.016	0.019	0.008	0.015	0.025	0.031	0.030	0.021	0.020	0.009	0.025	0.022	0.010	0.008	0.016	0.015	
***P. trifoliata*.AB505932**	0.007	0.007	0.008	0.007	0.011	0.012	0.010	0.013	0.007	0.009	0.021	0.029	0.028	0.019	0.023	0.003	0.022	0.019	0.006	0.006	0.019	0.019	0.009

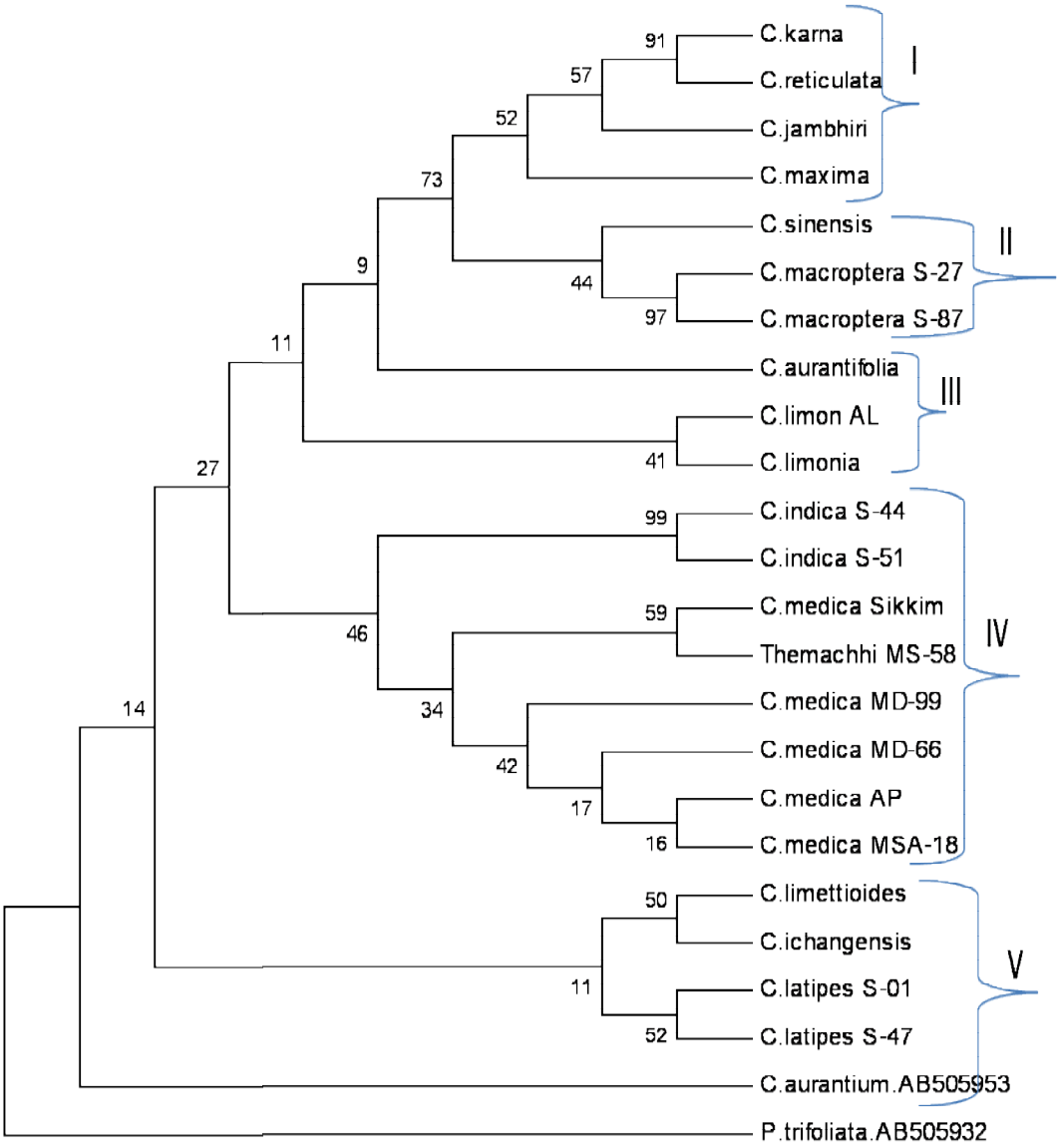

Figure 4.17: MP bootstrap consensus tree of 23 accessions of *Citrus* and the out group, *Poncirus trifoliata* from *rbcL* sequence data analysis. Numbers are bootstrap values based on 500 re-sampling

6.4 Cryopreservation studies

6.4.1 Seed physiology

Fresh seeds of fifteen citron cultivars when kept for germination showed 70 to 100 % germination initially. 30 day old seedlings when scored for root and shoot length, gave seedling vigour in the range of 3.5 to 7.3 and vigour index in the range of 245 to 730 (Table 4.15).

Table 4.15.Germination and vigour index of *Citrus medica* cultivars

Sl. No.	Cultivar Name	Germination %	Seedling vigour		Vigour Index
			Root Length (cm)	Shoot Length (cm)	
1	Bemberia	80.00 (±0.04)	1.5	2.0	280
2	Themachi	70.00 (±2.72)	1.5	2.0	245
3	Pati Jora	100.00 (±0.00)	3.4	2.0	540
4	Bira Jora	100.00 (±0.00)	4.5	1.5	600
5	Soh-mondong	80.00 (±0.16)	2.8	2.1	392
6	Gondharaj	100.00 (±0.00)	3.0	2.8	580
7	Pongam-citron	90.00 (±0.02)	3.2	3.0	558
8	Holong Tenga	70.00 (±2.72)	1.5	2.0	245
9	Bore Tenga	90.00 (±0.02)	3.4	3.5	690
10	Jora Tenga	100.00 (±0.00)	2.5	3.0	550
11	Chonchuno	100.00 (±0.00)	2.5	1.3	380
12	Tayum	100.00 (±0.00)	3.8	3.5	730
13	Etrog	90.00 (±0.02)	2.6	1.8	306
14	Citron	80.00 (±0.16)	3.0	2.4	432

(Values in the parentheses indicate standard error of means)

6.4.2 Seed longevity and storage behavior

Fresh seeds of *C. medica* with initial moisture content ranging from 30-35 % showed 95-100%germination. When the seeds were dried to 14.82% moisture content (in silica gel for 17 hrs) and stored at room temperature they retained only 20% viability upto 30 days of storage, when stored at 5°C, retained 60 % viability upto 30 days and when stored at -20°C, retained 80% viability 30 days and after that viability drastically reduced to 10% (Table 4.16).

6.4.3 Cryopreservation of embryo

Fresh embryos of citron with moisture content 29.52 % retained 100% viability before cryopreservation and no recovery after cryopreservation. When the embryos were dried to 20-22% moisture content viability decreased to 80 %, the embryos with same moisture

content retained 40 % viability after cryopreservation. Viability percentage after cryopreservation was highest (70%) for embryos desiccated to 15.10 %. Further reduction in moisture content proved deleterious for the survival of embryos in *C. medica*.

6.4.4 Cryopreservation of embryonic axes

6.4.4.1 Air desiccation

The moisture contents and viability percentages of *C. medica* embryonic axes following desiccation under various time factors, with and without cryopreservation is given in Table 4.17 respectively. The initial moisture content was as high as 33.67 %. With increasing duration of desiccation, the moisture content of the axes declined steadily. A major portion of the moisture loss occurred during the first hour of desiccation (Table 4.17). However, there was not much difference in the moisture loss at 2^{nd} and 3^{rd} desiccation hours consequently. Since the recovery growth of fresh, desiccated and frozen axes was rapid and normal on culture media 'A' in comparison to media 'B', so observations using only the former are reported. Reduction in moisture content of axes was, however, accompanied by a reduction in the survival rate. The initial viability values (at 0 hr desiccation) of embryonic axes were about 100%. The viability of axes declined steadily with increasing duration of desiccation (Table 4.17). The viability remained as high as 90% at 23.71% moisture content and declined to 40% at 7.49% moisture content. It was seen that viability fell drastically after 4 hours of desiccation at moisture content below 14.71%. Viability was completely lost when the tissue was brought below this moisture level. Cryopreservation was efficient for the axes desiccated to 14.71% and lower moisture content. Freezing of embryonic axes at 14.71% moisture level in LN at 196˚C showed a good recovery rate (66.67%), whereas embryonic axes possessing moisture contents above 23.71% and below 7.49% lost viability completely when exposed to LN (Table 4.17).

Table 4.16: Viability of embryos of Themachi and Tayum cultivars of *C. medica* at various temperature regimes

Cultivars	MC%		Storage temp.	Viability% after storage				
	Fresh	DC		0day	15days	30days	60days	90days
Themachi	49.26	14.82	RT	95.00(±2.72)	20.00 (±2.36)	20.00(±2.36)	00.00(±0.00)	00.00(±0.00)
	(±0.19)	(±0.17)	5ºC	95.00(±2.72)	60.00 (±0.16)	60.00(±0.04)	10.00(±0.16)	00.00(±0.00)
			-20ºC	95.00(±2.72)	80.00 (±0.04)	80.00(±0.06)	10.00(±0.16)	10.00(±2.36)
Tayum	47.28	15.23	RT	100.00(±4.76)	20.00(±0.11)	00.00(±0.00)	00.00(±0.00)	00.00(±0.00)
	(±0.26)	(±0.11)	5ºC	100.00(±4.76)	80.00(±1.16)	80.00(±2.72)	10.00(±2.41)	10.00(±0.06)
			-20ºC	100.00(±4.76)	100.00(±2.36)	100.00(±2.42	20.00(±2.08)	20.00(±0.04)

Table4.17: Survival of embryonic axes of Citron after different periods of desiccation and LN exposure

Desiccation duration (hrs)	Moisture content (%)	Viability (%) Before LN exposure	After LN exposure
0	33.67 (±0.47)[a]	100.00 (±0.00)[a]	0.00 (±0.00)
1	27.57 (±0.84)[b]	100.00 (±0.00)[a]	0.00 (±0.00)
2	23.71 (±0.21)[c]	90.00 (±4.71)[b]	25.00 (±2.36)[d]
3	19.44 (±0.20)[d]	85.00 (±2.36)[b]	50.00 (±2.36)[b]
4	14.71 (±0.26)[e]	75.00 (±2.36)[c]	66.67 (±0.63)[a]
5	9.48 (±0.15)[f]	60.00 (±2.36)[d]	33.33 (±2.36)[c]
6	7.49 (±0.03)[g]	40.00 (±2.36)[e]	16.67 (±0.16)[e]

Values in the parentheses indicate the standard error of means. Means with the same letter (superscript) in the columns showing viability before and after liquid nitrogen exposure do not significantly differ (P = 0.05) based on Duncan test.

6.4.4.2 Vitrification

Pre-culture of embryonic axes for 24 hrs in pre-culture medium retained 100% viability in *C. medica*. Treatment with loading solution for 20 minutes without cryopreservation retained 90% viability, but after cryopreservation there was only 25% viability. Exposure of axes to PVS2 for 20, 40, 60 and 90 min without LN exposure gave 85, 83.33, 75 and 55% viability, while the same treatment after LN exposure showed 33.33, 75, 50 and 16.67% viability, respectively (Table 4.18). Thus, after vitrification with PVS2 treatment for 40 minutes followed by cryopreservation in LNwas found to be optimal for axes survival.

Table4.18: Survival of embryonic axes of Citronafter vitrification

Treatment	Viability (%) Before LN exposure	After LN exposure
PMV	100.00 (±0.00)[a]	0.00 (±0.00)
LS	90.00 (±2.36)[b]	25.00 (±2.36)[d]

PVS2 20	85.00 (±0.61)[b]	33.33 (±2.72)[c]
PVS2 40	83.33 (±2.36)[b]	75.00 (±2.36)[a]
PVS2 60	75.00 (±2.36)[c]	50.00 (±2.36)[b]
PVS2 90	55.00 (±2.36)[d]	16.67 (±0.25)[e]

Values in the parentheses indicate the standard error of means. Means with the same letter (superscript) in the columns showing viability before and after liquid nitrogen exposure do not significantly differ (P = 0.05) based on Duncan test.

6.4.4.3 Encapsulation-dehydration

Fresh encapsulated axes showed high viability (100%) when cultured *in vitro* (Table 4.17). Encapsulated axes following 40h of pre-culturein various sucrose concentrations *viz*., 0.3M, 0.5M, 0.75M and 1.0M had moisture contents of 82.50, 78.46, 74.65 and 69.45% respectively. Afurther 6 hour of air desiccation reduced the moisture contents to less than 20%, but wasaccompanied by a decline in viability. Pre-culturingbeads on 0.5M and 0.75M sucrose followedby 6 hour air desiccationgave comparable results on survival of axes before and aftercryopreservation (Figure 4.21). But successful cryopreservation with highest viability (60%) was achieved foraxes pre-culturedfor 40h in 0.5M sucrose pre-culturemedia, followed by 6h desiccation treatment (Table 4.19, Figure 4.22 d).

Table4.19: Survival of embryonic axes of Citron after encapsulation-dehydration and freezing

			Viability (%)	
Treatment	**Moisture content (%)**	**MC % after 6 hrs AD**	**Before LN exposure**	**After LN exposure**
PME	-	-	100.00 (±0.00)[a]	0.00 (±0.00)
0.3 M	82.50 (±0.47)[a]	19.54 (±0.12)[a]	80.00 (±2.36)[b]	40.00 (±2.36)[c]
0.5 M	78.46 (±0.21)[b]	15.58 (±0.09)[b]	75.00 (±2.36)[b]	60.00 (±2.36)[a]
0.75 M	74.65 (±0.25)[c]	12.60 (±0.24)[c]	74.10 (±0.33)[b]	50.00 (±2.36)[b]
1.0 M	69.45 (±0.22)[d]	11.83 (±0.08)[d]	50.00 (±2.36)[c]	33.33 (±1.36)[c]

Values in the parentheses indicate the standard error of means. Means with the same letter (superscript) in the columns showing viability before and after liquid nitrogen exposure do not significantly differ (P = 0.05) based on Duncan test

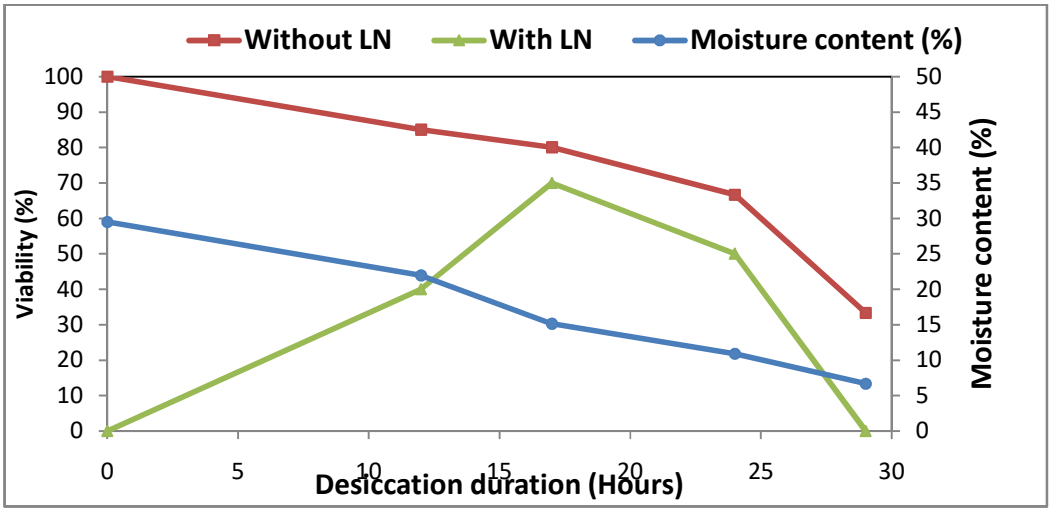

Figure 4.18: Viability of embryonic axes of Citron after different periods of desiccation and LN exposure

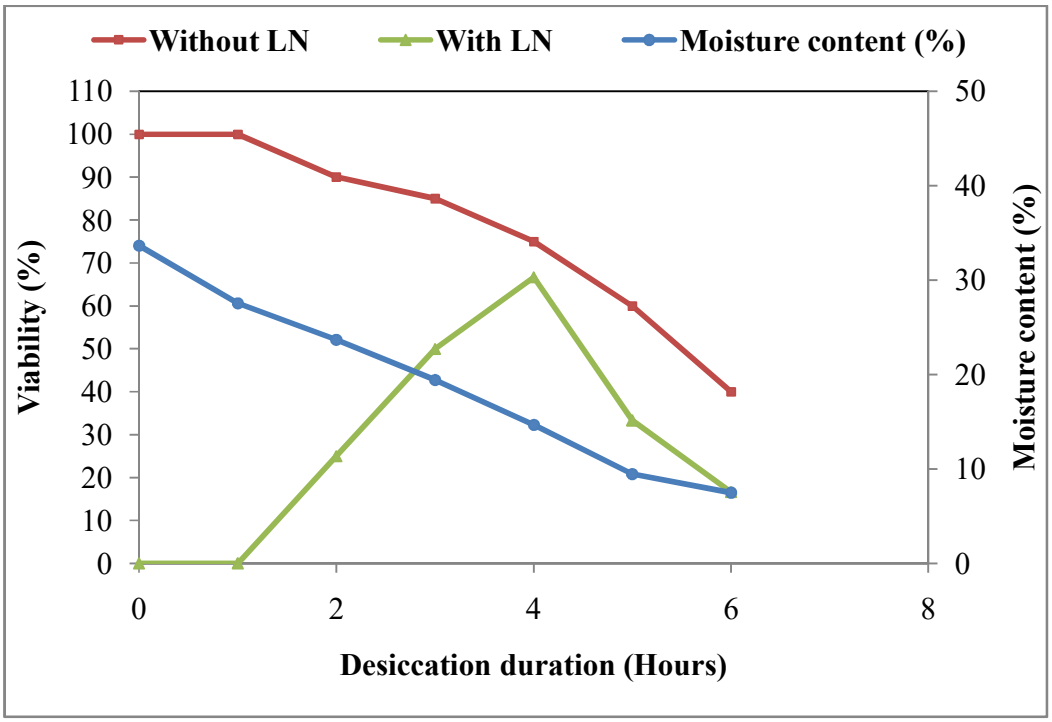

Figure 4.19: Survival of embryos of Citron after different periods of desiccation and LN exposure

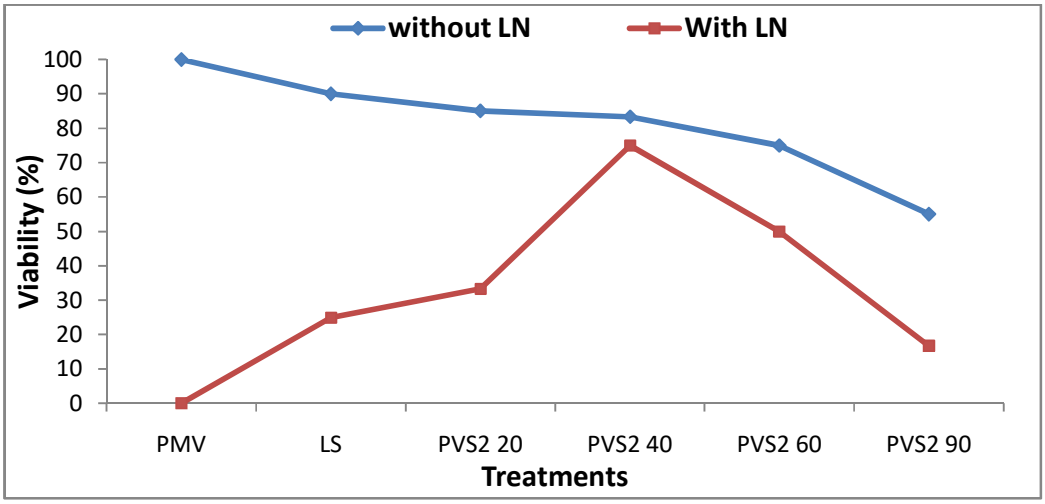

Figure 4.20: Survival of embryonic axes of Citronafter Vitrification

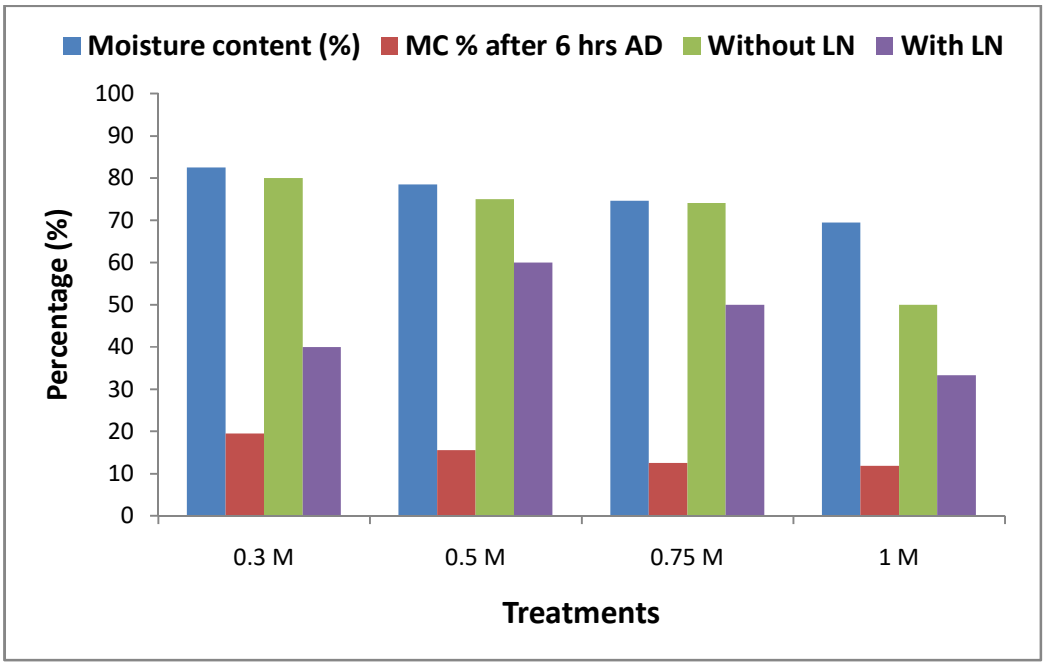

Figure 4.21: Survival of embryonic axes of Citron after encapsulation-dehydration and freezing

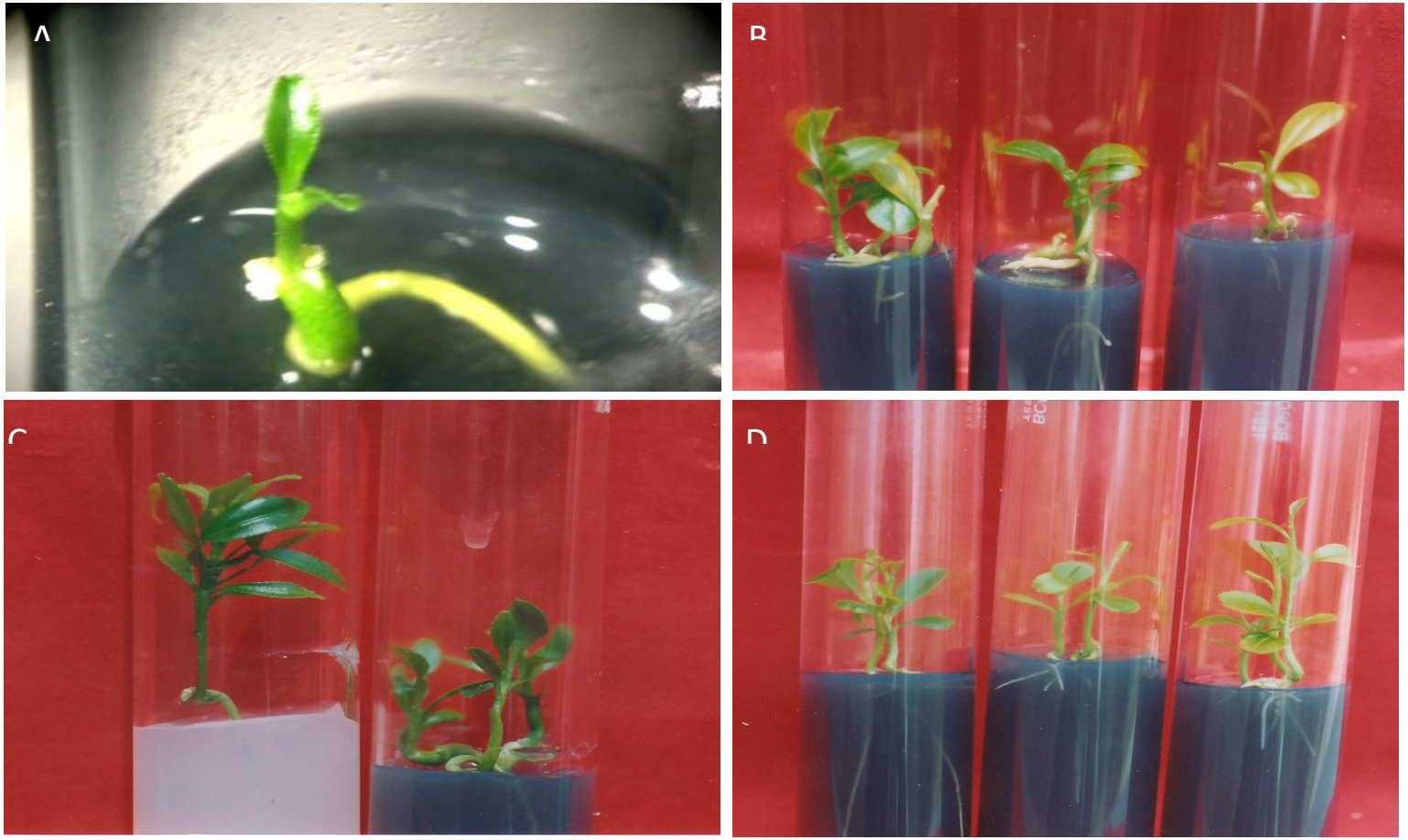

Figure 4.22 : Plant regeneration from embryonic axes of *C. medica* after 3 weeks of culture (a) Fresh embryonic axes regeneration showing root and shoot (b) after Air desiccation (4h + LN exposure), (c) after vitrification (40 min LS+ LN exposure) and (d) encapsulated beads (0.5 M sucrose + LN exposure) after cryopreservation.

CHAPTER V

DISCUSSION

Citrus is a diverse genus with species having vast phenotypic variations in tree and fruit characters. Some of the major citrus fruit crops of the world are, Citron[*C. medica* L.], Lemon [*C. limon*(L.) Burm.*f.*], Lime [C. *aurantifolia* (Christm.) Swingle], Mandarin [*C. reticulata* Blanco], Sour orange [*C.aurantium* L.], Sweet orange [*C.sinensis* (L.)Osbeck], Pummelo [*C. maxima*(Burm.)Merr.], Grapefruit [*C. paradise* Macf.] and Kumquats [*C. microcarpa* Bunge] (Swingle and Reece, 1967; Mabberley 1998, 2004).

E. Bonavia, a Brigade Surgeon of Indian Medical Services in the year 1988 in his book entitled "The Cultivated Oranges and Lemon etc. of India and Ceylon" gave a historical account describing almost each Citrus type present in India during that period and also enumerated the historical importance of each Citrus fruit, their cultivation and important characters.Among this many *Citrus* species, *C. medica* (Citron) is one of the important species of genus *Citrus*having high socio-economic, cultural and religious importance in India and several other countries. This species also have important place in citrus taxonomy and important role in origin and evolution of various other citrus types. The citron (*C. medica* L.) was the first citrus fruit, which was brought from its native habitat in India or Indo-China to Europe about 300 BC by Alexander (Webber, 1967). Since ancient times, citron has been used mainly for medical purposes such as to cure sickness, intestinal ailments, scurvy, pulmonary troubles and various other diseases. The citron is used by the Jews (known as Etrog in Hebrew) for a religious ritual during their "Feast of Tabernacles". Besides this, citron has high Vitamin C content and is used medicinally for treating conditions such as nausea, vomiting, skin diseases, excessive thirst and weak eyesight (Mabberley 1998, 2004).

In India, this is one of the most diverse *Citrus* types being known in all most all the parts of country, however, by the different names and diverse uses. Therefore, study of plant genetic resources of this important species is of great relevance in Indian context.

Characterization of plant genetic resources and quantification of diversity is essential for identification of species and cultivars, deciphering genetic relationships including parentages and for efficient management of germplasm. Lack of information on

specific plant characters and overall genetic diversity is one of the principal constraints for a wider use of germplasm collections by plant breeders and horticulturists. Germplasm characterization helps in unambiguous discrimination between accessions, identification of economically important plant resources, detection of redundancies and in monitoring genetic changes during maintenance. Estimates of diversity are useful to guide collection missions, monitor genetic erosion, establish core subset, select suitable parents for breeding programmes and to develop *in situ* and *ex situ* conservation strategies. Such studies are especially lacking in the tropical fruits growing as wild and semi-wild in the North-Eastern region of country.

The fundamental missions of the PGR manager is to preserve as broad a sample of the extent of genetic diversity as is scientifically and economically feasible (Patterson, 1996). Information on the extent of diversity of a species is thus, a basic necessity. Traditionally, germplasm has been classified on the basis of morphological and agronomic traits, but recently the use of molecular markers to study diversity and characterization in plants has become common. In the present study, the characterized accessions were mainly grouped according to leaf, fruit and seed morphological characters, which are complex and multigenic characters. Such characters are environmentally affected and therefore, liable to transform during evaluation. In this sense, the molecular characterization is more efficient in the generation of an unbiased picture of diversity than agronomic approach. However, the agronomic characterization is still important in germplasm management, and determination of molecular diversity should not be seen as replacing traditional characterization methods but rather as a complement to it.

In the present study, *C. medica* an important citrus fruit species which is believed to be native to India, particularly, the North-Eastern India has been taken up for genetic resource management. Comparison of morphological and molecular characterization data is of immense importance to conclude the extent of genetic diversity present in the set of accessions. Although the correlation between the morphological and molecular data was low in the analyzed accessions of *C. medica*, both methods allowed fare groupings of accessions based on the analyzed traits and area of collection. Despite the fact thatmorphological traits were relatively less efficient for precise discrimination of closely related genotypes, the cost and time invested were lower than for molecular analysis. Moreover, phenotypic expression of important characters of genotypes has more importance for the selection of suitable genotype for

desired characters in the commercially valued fruit species. Therefore, based on cost, efficiency and practicality the information gained by molecular characterization complements the morphological characterization in *C. medica* and provides valuable information for selecting commercially viable and superior genotypes. In the present study, "Bemberia" genotype from Sikkim and "Tayum" genotype from Arunachal Pradesh were found to be superior and promising types and could be used as mother plants for generating quality planting material for farmers.

Although the characterization of citrus germplasm for various species has been documented by several works, this is the first time that *C. medica* accessions collected from different parts of India were characterized using morphological and molecular markers. The results of this study effectively identified the potential intra and inter accessions genetic variation in *C. medica* within a closed agro-ecological region of its distribution. It has further showed the advantages of using molecular markers in revealing genetic diversity present in this species and using the same for selection of superior genotypes and crop improvement studies of this emerging and potential fruit crop of India.

5.1 Genetic diversity analysis

Currently, several powerful marker techniques are available for genetic analysis of both plant and animal species. The choice of the most appropriate markers and techniques principally depends on the objective of research and the biology and genetic structure of the species. Therefore, comparisons are desirable in order to fix on the most appropriate technique for the issue being examined. In this study, genetic diversity in 46 accessions of *C. medica* collected from different parts of India was assessed using RAPD, ISSR and SSR markers. This study has demonstrated the potential of RAPD, ISSR and SSR markers as a rapid, reproducible and useful method for distinguishing different accessions of *C. medica* and for clustering of the accessions into different groups. RAPD (17 primers), ISSR (11 primers) and SSR (16), were sufficient to provide significant information on the genetic diversity within *C. medica*.

RAPD, ISSR and SSR data generated from 46 accessions of Citron with 17, 11 and 16 primers, respectively, were sufficient to provide inferences on genetic differentiation and relationships among the accessions. In the 46 accessions of *C. medica*, moderate level of polymorphism (78.79%) was estimated by SSR markers and ISSR markers (73%) while RAPD markers revealed moderately high level of

polymorphism (82%). The variations in the level of polymorphism generated by these three markers are due to different target regions in the genome. RAPD primer targets entire genome including coding and non-coding regions as well as repeated sequences, while ISSR primer targets only region between two microsatellite loci and SSR primers target only specific region in the genome.

A high level of polymorphism (86%) was also reported by Campos *et al.* (2005)in mandarins based on AFLP markers. Although, ColettaFilho*et al*.(1998) reported very narrow genetic base of mandarin group using RAPD marker and proposed that the mandarin group was a single species,*C. reticulata*. PIC values were also recorded high in both RAPD (0.661) and ISSR (0.664) markers, showing the efficiency of molecular marker used to detect polymorphism within the mandarin group. In the present study, PIC value (0.332) of ISSR markers were observed higher in comparison to RAPD(PIC= 0.297) and SSR markers (PIC= 0.2965). Similar results were also reported by Pal *et al*. (2013). In the present study, RAPD markers were found to be more efficient than SSR and ISSR assay in respect to polymorphism detection, as they detected higher polymorphism (82%) as compared to SSR (78.79) and ISSR (73%) markers along with the higher average number of polymorphic loci per primer for RAPD followed by SSR and ISSR markers. However, SSR marker could detect greater total heterozygosity (Ht = 0.4174), while, ISSR marker had greater Shannon's information index (I= 0.475) than RAPD, i.e. Ht = 0.257 and I= 0.400, respectively. Genetic diversity parameters like average and effective number of alleles, percentage of polymorphism, polymorphic information content (PIC), effective marker index (EMI), and marker index (MI) parameters for RAPD, ISSR, SSR and combination of RAPD +ISSR+SSR revealed that these parameters were significantly higher when using RAPD markers.

Jaccard's genetic similarity value was found in the range of 0.42 to 0.97 (average 0.70), 0.38 to 0.99 (average 0.69), 0.49 to 0.49 to 0.96 (average 0.73) and 0.49 to 0.96 (average 0.73) in SSR, ISSR, RAPD and combined data analyses, respectively. Genetic similarity values analyzed among all the 46 accessions of *C. medica* through RAPD, ISSR and SSR markers, individually as well as in combination, showed significant level of genetic variation within *C. medica* (RAPD, ISSR, SSR and Combined). This inference is correspondingly congruent with the values of total heterozygosity and Shannon's information index of *C. medica* generated through

RAPD, ISSR and SSR markers individually as well as in combination. The resolution of combined dendrogram was comparatively higher than the separate RAPD, ISSR and SSR dendrogram in term of genetic relationship.

UPGMA dendrogram drawn from RAPD data also supports the clustering pattern of ISSR and SSR data, while in all the cases no geographic isolation of *C. medica* accessions is shown. Grouping of 46 accessions of *C. medica* was almost similar in all the four dendrograms (RAPD, ISSR, SSR and Combined) except few transitions like MD-17 (Bemberia) grouped along with MD-22 (Bemberia) in cluster I in ISSR dendrogram,while in RAPD it formed a separate group within cluster I. MD-17 (Bemberia) formed a separate group in SSR in cluster I. One accession from Dibru-saikhowa, Assam (MD-11/83) formed a single cluster II in the ISSR dendrogram, but it segregated into a separate group within cluster I in the RAPD dendrogram. In SSR dendrogram, MD-11/83 formed a separate group along with MD-11/73 within cluster II.

A search for unique bands was made for all the accessions tested, in which a total of eighteen unique bands were generated in ten accessions by 11 RAPD primers, four unique bands in three accessions by 4 ISSR primers and six unique bands in five accessions by 6 SSR primers. In accession no. MD-22 (Bemberia), a maximum of two unique bands was given by primer OPD-20 (820bp, 420bp) and OPF-03 (470bp, 450bp). Similarly, unique bands were generated in accession MD-17 (Bemberia), MD-22, MD-11/49 (PatiJora), MD-154 (Themachi), MD-11/65 (Pongam citron), MS-42 (Themachi), MAU-01, MAU-06, MSA-18 (Etrog), CITRON-AP (Tayum), IPS/PNB/319, IC-202113 and IC-219067.These unique fragments can be used as a marker for identification of these cultivars, which will be useful for future conservation, maintenance and breeding programmes. These accessions can also be used for developing the core collection of *C. medica* germplasm.

5.2 Phylogenetic relationship analysis

Citrus classification is confusing and highly notorious. Various taxonomists have recognized 16 to 162 species in the genus *Citrus* (Swingle, 1943 and Tanaka, 1958). Most of the puzzlement is due to free hybridization of different species and incidences of intermediate forms. The cpDNA sequences are the primary source of characters for phylogenetic studies in plants (Small *et al.,* 2005; Bayer *et al.,* 2009). Protein-coding gene sequences such as *rbcL* and *matK* have been used to elucidate phylogenetic relationships among higher-level taxa (Chase *et al.,* 1993; Penjor*et al*.,

2010; 2013). Subsequently, the potential utility of non-coding regions of the chloroplast genome was recognized for lower-level studies (Taberlet*et al.,* 1991; Jung *et al*., 2003). Recently, Lu *et al.* (2011) investigated the molecular phylogeny of 30 genotypes from six genera of the true citrus fruit trees by conducting research on three cpDNA regions. In another report, Morton *et al.* (2003); Jung *et al*., (2005) andJena *et al*. (2009) carried out molecular phylogeny in Indian *Citrus* L. (Rutaceae) based on *trnL-trnF* sequence data of chloroplast DNA. Wali*et al*., (2013) studied the phylogenetic relationships on selected *Citrus* species based on chloroplast gene, *rps14*. Earlier, with the help of cpSSR Cheng *et al.* (2005) and Deng *et al.* (2007) have reported the molecular phylogeny of *Citrus*. ITS sequence has also been used for inferring the phylogenetic relationship in *Citrus* species by many workers (Kyndt*et al*., 2010; Kumar *et al*., 2012; Amar *et al*., 2014; Hynniewta*et al*., 2014; Sun *et al*., 2015).

In the present study, *rbcL* and *matK* gene sequence analyses of the cpDNA was used to investigate the phylogenetic relationship of Indian citron with other important commercial *Citrus* spp. In our studies, *C. maxima*, *C. medica and C. reticulata* were separated in distinct groups or sub-clusters which supports their distinctiveness as the true basal species of edible Citrus. This concept has gained much acceptance and support through recent morphological, biochemical and molecular studies conducted by differentcitrus taxonomists (Scora, 1975; Barrett and Rhodes, 1976; Nicolosi*et al*., 2000; Arau'jo*et al*., 2003; Mabberley, 2004; Liang *et al*., 2007; Pang *et al*., 2007; Jena *et al*., 2009).

C. medica (Citron) is one of the basic species of Indian origin and is believed to have acted as male parent in the origin of several hybrids/cultivars of *Citrus* such as all true lemons and rough lemon (Barrett and Rhodes 1976; Federici*et al*., 1998; Nicolosi*et al*., 2000; Gulsen andRoose 2001; Moore 2001; Mabberley 2004). Our *rbcL* and *matK* sequence data recognized *C. medica* as a true basic species as both wild and domesticated accessions of the species grouped in the cluster I with a very high bootstrap value of 87% (NJ tree) and 88% (MP tree). Citron, as an important true species, took part in the origin of many *Citrus* species, but our cpDNA data analysis indicates that citron has always acted as the male parent (Nicolosi*et al*., 2000).

C. maxima (Pummelo)and *C. reticulata* (Mandarin) are believed to have contributed to the development of several commercial citrus fruits, such as sour orange (*C. aurantium*, a cross between mandarin and pummelo), sweet orange (*C. sinensis*) (L.)

Osbeck (a backcross between pummelo and mandarin), grapefruit (*C. paradisi*) (a backcross between pummeloand sweet orange) (Moore, 2001; Mabberley, 2004). Pummelo was reported as one of the three true *Citrus* species by Barrett and Rhodes (1976) and most of subsequent studies were in agreement with this statement (Federici*et al*., 1998; Nicolosi*et al*., 2000; Barkley *et al*., 2006; Uzuan*et al*., 2009). Pummelo has played an important role as a parent of many citrus fruits, such as lemons, oranges and grapefruits. *C. maxima* clustered with papedas, particularly with *C. latipes*. In UPGMA tree, the sour and sweet oranges were grouped together in a separate cluster along with the Khasi papeda and Melanesian papeda, while in the MP and NJ trees, the grapefruit and sour orange formed a separate cluster along with *C. reticulata*, and the sweet orange grouped with *C. maxima* along with the Khasi papeda and Melanesian papeda. The consistent grouping of sweet orange with *C. maxima* in the *rbcL* and *matK* derived trees indicates the role of *C. maxima* as a male parent in the origin of sweet oranges.

C. indica (Indian Wild Orange) is a true wild species endemic to the Garo hills in Meghalaya. Tanaka (1928) was the first to describe it as a new species. Swingle and Reece (1967), however, suspected *C. indica* to be of hybrid origin involving a wild species of *Citrus* (*C. latipes*?) and one of the cultivated species of *Citrus* as putative parents. Therefore, elucidating its special taxonomic position as a true species or progenitor species of cultivated *Citrus* taxa. *C. medica* (citron), *C. reticulata* (mandarin) and *C. maxima* (pummelo) are defined as basic true species by Swingle and Reece (1967) a phylogenetic truth which was later supported by a number of workers (Barrett and Rhodes, 1976; Jena *et al*., 2009; Kyndt*et al*., 2010; Kumar *et al.,* 2012). *C. indica* accessions clustered with *C. medica* in both *rbcL* and *matK* sequence databased on NJ and MP trees. Similar clustering pattern was reported earlier by Nicolosi*et al.* (2000), Federici*et al*. (1998) and Jena *et al*. (2009) based on PCR-RFLP of cpDNA. Mabberley (2004) also subscribed Swingle's view in treating *C. indica* as a species of suspected hybrid origin. Based on RAPD and PCR-RFLP data, Federici*et al*. (1998) argued against the hybrid origin of *C. indica*. Our studies also do not support the hybrid origin of *C. indica* as it consistently separated out as a distinct group along with *C. medica*.

C. aurantium (sour orange) is considered as a hybrid, a cross between *C. reticulata* (Mandarin) and *C. maxima* (Pummelo). In our study, we found that *C. aurantium* clustered with *C. reticulata*. Thus, our data support the role of Mandarin as

one of the maternal parents in the hybrid origin of *C. aurantium* (Jena *et al*., 2009; Kumar *et al*., 2012).

C. jambhiri (rough lemon) is considered as a hybrid originated from *C. medica* and *C. reticulata* (Scora, 1975; Barrett and Rhodes, 1976; Nicolosi*et al*., 2000; Mabberley, 2004). Based on the UPGMA obtained through NJ tree and MP tree, *C. jambhiri* was found to be clustered with *C. reticulata*. Our data thus show a close relationship between *C. reticulata* and *C. jambhiri* and support the role of *C. reticulata* as maternal parent in the hybrid origin of *C. jambhiri* (Federici*et al*., 1998; Nicolosi*et al*., 2000; Barkley *et al*., 2006).

C. sinensis loosely clustered with *C. maxima* and *C. reticulata* in our *rbcL* sequence data. It's clustering with *C. maxima* in the *rbcL* NJ tree indicates the hybrid origin of *C. sinensis* involving *C. maxima* as one of the putative parents, and thereby supporting the views of Barrett and Rhodes (1976), Luro*et al*. (1995) and Nicolosi*et al.* (2000).

Several earlier workers hypothesized *C. limon* to be of complex hybrid origin involving two parents: citron and lime (Swingle, 1943; Malik *et al*., 1974; Scora, 1975) or citron and sour orange (Nicolosi*et al*., 2000; Gulsen and Roose, 2001) or sour orange and lime (Hirai and Kozaki, 1981; Torres *et al*., 1978). Most lemons have highly similar morphological and biochemical characters, and some are reported to have originated by mutation from a single parental lemon tree. In our study, *C. limon* grouped with *C. limonia*, *C. aurantifoli*a and *C. macroptera*based on cpDNA data. This study showed that *C. aurantifolia* (sour lime) is involved as one of the parents in the origin of *C. limon*.

C. aurantifolia waproposed as a trihybrid origin, involving citron, pummelo and a species of *microcitrus* in the parentage (Barrett and Rhodes, 1976)). RFLP data of Federici*et al*. (1998) supported citron as one of the parents involved in the origin of *C. aurantifolia*. In our data based on *rbcL* NJ tree, *C. aurantifolia* was found to be closely related to *C. limon* and *C. limonia.* It was loosely clustered with *C. medica* suggesting the role of Citron as one of the maternal parents involved in the origin of *C. aurantifolia.*

Karna orange or Karna khatta (*C. karna*) has long been known in India and exploited as a root stock for grafting commercial Citrus varieties. Fruit characters of *C. karna* show resemblances with *C. aurantium, C. medica*, and *C. maxima*. In our cpDNA analysis, *C. karna* consistently found a place along with other taxa of suspected

hybrid origin. In our study based on *rbcL* sequence data, *C. karna* was found to be closely related with *C. reticulata* and *C. maxima* which suggests the involvement of either *C. reticulata or C. maxima* as one of the maternal parents in the origin of *C. karna*. However, there is no conclusive evidence to elucidate the mode and actual parentage involved in the origin of *C. karna*.

C. limettioides was supposed to have originated as a hybrid of *C. aurantifolia* with *C. limetta*Risso or with a sweet citron (*C. medica* var. dulcisRissoetPoit) (Webber, 1943) or a cross between *C. aurantifolia* with *C. sinensis* (Barrett and Rhodes, 1976). The cpDNA profiling by Nicolosi*et al*., (2000) could not trace the parents involved in the origin of *C. limettioides*, although a SCAR analysis by the same authors indicated citron and sweet orange as putative male and female parents, respectively, of the Indian sweet lime. In our study, *C. limettioides* was found to be closely related with *C. sinensis* based on *matK* NJ and MP tree. This suggests that, *C. sinensis* may be one of the possible parents of *C. limettioides* (Barrett and Rhodes, 1976).

*C. macroptera*commonly called as Melanesian papeda has wide spread distribution in India especially in the North-Eastern part of India as compared to other endangered *Citrus* species. The fruits are being very juicy and vesicles very small resembling that of the lime. Swingle and Reece (1967) considered it as a promising rootstock and useful for breeding new rootstocks. In our study, *C. macroptera* clustered together with *C. sinensis* on the basis of *rbcL* sequence data and it clustered with *C. aurantiifolia* and *C. limon* based on *matK* gene sequence in both NJ and MP trees. Our data infer close genetic relationship between this species and their probable origin from the same genetic lineage.

C. latipes (Khasi Papeda) is known to have originated in India probably in the North-Eastern part of India (Bhattacharya and Dutta, 1956). The fruit being inedible have little commercial value. It has been tried as a root-stock for the Khasi orange (*C. reticulata*) and is found to be incompatible. Tanaka (1977) hypothesizes that *C. latipes*may have originated from *C. maxima*. The cpDNA data in our studies support this hypothesis. The presence of *C. latipes*in the pummelo cluster might indicate that the ancient maternal relationship is in the cluster. The cpDNA profiling by Nicolosi*et al*., (2000) also supported Pummelo as the maternal parent of *C. latipes*.

C. ichangensis (Ichangpapeda) is not a cultivated fruit and is absolutely inedible. It is reported to be very much cold-resistant. The fruits practically contain no juice and have no commercial importance. Its value as a root-stock has not yet been

ascertained. Major differences exist between Swingle's (1943) and Tanaka (1977) systems regarding the taxonomy of *C. ichangensis*. Swingle placed it in the subgenus*Metacitrus*, which contained all Mandarin species and some hybrids of *C. ichangensis* but no other*Papeda* species at all. Zhu (1988) showed that *C. ichangensis* was a primitive *Citrus* species. Herrero *et al*, (1996) found that isozyme data clustered *C. ichangensis* with *C. karna* and *C. meyeri*, which are lemon types. The analysis of Fraction I protein conducted by Handa*et al*. (1986) showed that *C. ichangensis* obviously differs from the other *Papeda* species which originated in tropical or subtropical regions by its cold hardiness and having single flowers. The present studies based on sequence data of cpDNA results show that *C. ichangensis* is a distinct species very different from most other *Citrus* species (Federici*et al.,* 1998; Nicolosi*et al*., 2000; Pang *et al*., 2007).

Swingle and Reece (1967) had divided citrus into two subgenera: *Citrus* and *Papeda*. The members of subgenus *Papeda* are distinguishable from subgenus *Citrus* in having large sized fruits containing acrid oil droplets in their pulp-vesicles; leaflets with broadly winged petioles that are usually as long or longer than the leaflet blades; free stamens; presence of purplish tinged on new shoots and flowers; and a epigeous mode of seed germination. However, our cpDNA analysis based on *rbcL* and *matK* gene sequence, could not find any clear cut differentiation between subgenera *Citrus* and *Papeda*. This supports the earlier findings of earlier workers (Nicolosi*et al*., 2000; Pang *et al*., 2007; Jena *et al*., 2009; Kumar *et al*., 2012;Hynniewta*et al*., 2014).

5.3 Cryopreservation

Ascertaining seed storage behavior of cultivar or species is important before adopting any*ex-situ* conservation approach. The success of *ex-situ* conservation depends on longevity of seedsunder storage and the ability to generate the whole explants /plantlet after retrieval from the storage conditions. Seed longevity (i.e. the period of survival) varies greatly among species. It may also vary the accessions within a species because of differences in genotype and provenance. Seeds of *Citrus* are classified into intermediate category i.e. these seeds with large size and high moisture content at the time of shedding are variably desiccation and freezing sensitive (Ellis *et al*., 1990; 1991; Hong and Ellis, 1996).

Substantial loss of seed viability in *C. medica* at ambient, 5^{o}C, and 20^{o}Ctemperatures within 30-60days of storage period confirms the desiccation and freezing sensitivenature of seeds of these cultivars. Sharp decline in the viability of seeds at

ambient temperaturewithin 15 days confirms the short longevity of these cultivars. These studies confirm theintermediate seed storage behavior of citron (Malik *et al*., 2006).

Excised embryonic axes are preferred because of their small and organized structure,independent identity and appreciable proportion of meristematic tissues with high morphogeneticpotential, moreover, they have the ability to produce vigorous plantlets by careful manipulationof post-freezeconditions, and represent good material for cryopreservation (Chaudhuryand Malik, 1999).

Cryopreservation techniques have found application in the long-termconservation of*Citrus* germplasm using seeds, embryonic axes and somatic embryos. Viability percentages of 50to 100% have been achieved using air desiccation (Normah *et al*, 1997; Radhamani andChandel, 1992; Cho *et al.,* 2002; Malik and Chaudhury, 2006; Malik *et al*., 2012), pre-growthdesiccation (Cho *et al.* 2002), vitrification (Cho *et al.,* 2001a, b; Malik and Chaudhury, 2006)and encapsulation dehydration (Cho *et al*., 2002; Malik and Chaudhury, 2006; Malik *et al*., 2012).

The desiccation sensitivity of an explant is the degree of its tolerance to lose free waterwithout associated damage and a decline in viability. In this experiment we have demonstratedthe desiccation sensitivity of embryonic axes of *C. medica* at distinct rates of drying. Theembryonic axes had high initial moisture content. A relatively high critical moisture content (level below which significant reduction in viability is recorded), a significant decline in viability with the reduction in moisture content, and sensitivity to freezing, are indicative of desiccationand freezing sensitive nature of embryonic axes. Successful cryopreservation using airdesiccationfreezingwas achieved in the embryonic axes of *C. medica.*

A vitrification technique used to freeze apices of a range of species is being usedwith variable success to freeze embryonic axes of some intermediate and recalcitrant species*viz*.,neem(Chaudhury and Malik, 1999), rubber(Sam and Hor, 1999) and jackfruit(Thammasiri,1999). Using this technique in *Citrus* species, a recovery rate of 62.5% was reported in *C.sinensis* (Sudarmonowati, 2000) and 82.5% in *C. madurensis*(Cho *et al.,* 2001b). In the presentstudy, vitrification was successfully applied leading to high recovery value in *C. medica*.

During vitrification, the pre-cultureof explants on sucrose or sorbitol enriched mediumfor 1 or 2 days has shown to induce dehydration tolerance, thereby improving the viability ofcryopreserved cells, meristems and embryonic axes (Thammasiri,

1999; Sakai, 2000: Cho *et al*., 2001b). The use of loading solution reportedly induces dehydration tolerance to freezedehydrationor to PVS2, and imparts protection against injury to membranes. The beneficialeffect of loading has been reported for several tropical monocots (Sakai, 2000), and was effectivein our experiments to enhance the recovery of vitrified axes.

Chemical dehydration with PVS2 before freezing affords better protection than simple airdehydration, as shown by the high percentage viability achieved after vitrification. The durationof PVS2 treatment is a further variable that can be manipulated to enhance the success ofcryopreservation (Sakai, 2000). Unlike shoot apices that are known to be sensitive to osmoticdehydration and/ or toxic chemicals, especially those used in vitrification solution, embryonicaxes are hardier and hence can more easily survive the various steps of vitrification.

The encapsulation-dehydrationtechnique has been applied to embryonic axes of *C.madurensis*(Cho *et al*., 2002) and *Heveabrasiliensis*(Yap *et al.*, 1999). The encapsulation-dehydrationtechnique has been reported for two *Citrus* rootstocks (*P. trifoliata* and citrange)(Engelmann *et al*., 1994), while an encapsulation-vitrificationprocedure has been developedwith the sour orange (*C. aurantium*) (Samia*et al*., 2002). In the present study, encapsulation-dehydrationprotocol was successfully applied leading to moderate recovery value of theembryonic axes of citron cultivars.

Chapter VI

Summary and Conclusion

India is the home of several *Citrus* species and has rich genetic diversity particularly in mandarins, sweet oranges, lime and lemons immensely contributed in improvement of citrus cultivation and industry in India. Besides this, rich diversity is represented in the form of wild and semi-wild species such as *C. indica, C. macroptera, C. medica, C. latipes, C. assamensis, C. ichangensis, C. megaloxycarpa*and *C. rugosa*are represented in several parts of India. North-Eastern India is thought to be the area of origin of several citrus species and abundant diversity of various citrus types is available in the North-Eastern states.

C. medica, (commonly known as citron) one of the important citrus fruits of the world, is considered a native of India and is found to grow in a wild and semi-wild state in primary as well as secondary forests along the foothills of the Eastern Himalayas and North-East India. Citron is monoembryonic in nature and is considered as one of the three basic species of *Citrus.* The citron is used mainly for medicinal purposes: to combat sea sickness, pulmonary troubles, intestinal ailments, and other disorders.

Natural population of *C. medica* is vanishing at alarming rate in the North-Eastern states and other parts of India due to lack of proper conservation and utilization, hence necessitating systematic survey, collection and characterization. Preliminary morphological characterization of Indian citron cultivars growing in North-East Indiahas been carried out by Ray and Deka, (1999)indicating significant variability within the species. However, extensive studies using molecular markers on the genetic diversity occurring in different parts of India has not been studied till now. There is an urgent need for studying the extent of diversity occurring in citron in different parts of India.

Although, several earlier workers had attempted to study and classify Indian *Citrus* from botanical as well as horticultural perspectives, confusion still abounds in proper identification, taxonomic disposition and circumscription of cultivated as well as wild and indigenous species/varieties of *Citrus* in India. The importance of *C. medica* in the ancestry of *Citrus* has been put into question mark. Such a geographically diverse species functioning as one of the parent of some present day

important cultigens suggests that the natural distribution of *C. medica* is not restricted to India. Due to this complex role and importance of *C. medica* as the basic species, there is an urgent need for studying the extent of diversity occurring in citron in different parts of India and their phylogenetic relationships with other closely related *Citrus* species and genera.

Cryopreservation has become a promising tool for long-term conservation of clonally propagated germplasm resources. It facilitates management of the *in vitro* collection by minimizing the risk of both somaclonal variation and contamination of cultures. Cryopreservation, offers a long-term storage capability, maximal stability of phenotypic and genotypic behaviour of stored germplasm, and minimal storage space and maintenance requirements. It has been considered to be an ideal tool for long-term storage of germplasm.

The present study, based on an extensive sampling from its natural habitat, is the first attempt to assess the genetic variability in Indian citroncultivars collected from different parts of India particularly the North-East India and also study the systematic and phylogenetic relationship of Indian Citron (*C. medica*) with other important *Citrus* species using *rbcL* and *matK* gene sequence of the chloroplast DNA (cpDNA). Thus, in the present proposed plan of work it was proposed to emphasize on following objectives.

1. Study of genetic variability existing in Indian Citron (*Citrus medica*)
2. Phylogenetic relationship studies of Indian citron in relation to other *Citrus* species
3. Cryobiology studies of seed, embryo and embryonic axes of *C. medica*

Specific exploration and collection missions were undertaken to different parts of India particularly the North-Eastern region of India viz., Assam, Arunachal Pradesh, Meghalaya, Nagaland, Sikkim, Himachal Pradesh, Uttarakhand and Abohar, Punjab for the survey and collection of *C. medica* germplasm. A total of forty six representative accessions of citron cultivars were collected from wild, semi wild and cultivated conditions for various studies. Collections were mostly made following selective sampling strategy, where samples collected from single plant was given an indigenous collection number (IC number) and treated as individual accession. Leaf and fruit samples of each accession were taken for confirmation of taxonomic identity, characterization and DNA extraction. Detailed information of each accession

was recorded in NBPGR passport database. Freshly extracted seeds were used for estimation of moisture content and viability. Desiccation and freezing sensitivity and longevity experiments were conducted on seeds and embryonic axes. Based on these studies seed storage behaviour was ascertained as intermediate for this species. Morphological characterization of leaves, fruits and seeds was undertaken using descriptors developed for Citrus by International Plant Genetic Resources Institute (IPGRI), Rome, Italy. Characterization data of 12 quantitative and 25 qualitative characters was recorded for the collected germplasm. Molecular characterization using RAPD, ISSR and SSR markers were used for characterizing and assessing the genetic diversity in the collected germplasm. Phylogenetic relationship of Indian citron with other important citrus genotypes was inferred based on *rbcL* and *matK* gene sequence analysis of chloroplast genome.

Cryopreservation techniques have been widely utilized for the long term conservation of citrus germplasm using seeds, embryonic axes and somatic embryos. The cryopreservation of embryonic axes of *C. medica* was attempted using air desiccation-freezing, vitrification and encapsulation-dehydration. Results of these three techniques have compared to ascertain the maximum survival of embryonic axes after liquid nitrogen exposure.

To conclude that the chloroplast DNA (cpDNA) analysis based on *rbcL* and *matK* sequence data carried out in Indian taxa of *Citrus* was useful in differentiating all the true species and species/varieties of probable hybrid origin in distinct clusters or groups. Sequence analysis based on *rbcL* and *matK* gene was able to provide unambiguous identification and disposition of true species like *C. maxima, C. medica, C. reticulata* and related hybrids/cultivars. The separation of *C. maxima, C. medica* and C. *reticulata* in distinct clusters or sub-clusters supports their distinctiveness as the basic species of edible citrus. The cpDNA sequence analysis of *rbcL* and *matK* gene could not find any clear cut differentiation between subgenera *Citrus* and *Papeda* according to Swingle's system.

This study shows that the *rbcL* and *matK* sequence data can detect genetic variation in Indian *Citrus* genotypes, but the utility of the data in inferring phylogeny at intra and interspecific levels is limited probably by factors such as hybridization, bud mutations, apomixis and polyploidy. However, this study was helpful in supporting the distinctiveness of *C. indica, C. latipes* and *C. ichangensis* as true species, besides elucidating the hybrid origin and relationships among the cultivated species/biotypes,

such as *C. aurantiifolia, C. limon, C. limettioides, C. aurantium, C. sinensis, C. karna* and *C. macroptera.* The outcome of this study will be further helpful in elucidating correct taxonomic identification, documentation, characterization and evaluation of Indian citron and utilization of its genetic resources in futurecrop improvement programmes.

Based on the above studies following conclusions were drawn:

Conclusions

1. This is the first study in India where genetic resources studies including morphological characterization, phylogenetic relationship and cryopreservation of Indian citron cultivars has been undertaken.
2. This study revealed significantly high morphological diversity among the collected *C. medica* germplasm.
3. Wide variations was observed for both qualitative and quantitative traits such as fruit weight, fruit size, fruit shape, fruit base, fruit apex, leaf lamina shape, leaf lamina margin, leaf apex, seed shape, cotyledon colour and chalazal spot colour.
4. The UPGMA dendrogram based on 37 morphological traits classified the accessions into two main clusters.
5. Principal components analysis performed on quantitative and qualitative traits revealed that the first three most informative components accounted for 46.02% variance.
6. This is the first study attempted to assess the genetic diversity of Indian citron germplasm using molecular markers.
7. This study effectively identified the potential intra-species genetic variation within a closed agro-ecological region of its occurrence.
8. Genetic diversity assessed by RAPD, ISSR and SSR markers revealed significant diversity in the citron accessions of India.
9. “Bemberia” biotype from Sikkim and “Tayum” biotype from Arunachal Pradesh were found to be genetically more diverse as compared to other cultivars.
10. Accession specific primers identified in this study can be used for further cultivar identification.

11. The placement of *C. medica* accessions in various sub-clusters and groups in the dendrogram was based on molecular differentiation of individual accessions rather than their geographical origin.
12. This study was the first to use both *rbcL* and *matK* gene sequence of chloroplast genome for inferring phylogenetic relationships in Indian citrus.
13. Sequence analysis based on *rbcL* and *matK* gene was able to provide unambiguous identification and disposition of true species like *C. maxima, C. medica, C. reticulata* and related hybrids/cultivars.
14. The separation of *C. maxima, C. medica* and *C. reticulata* in distinct clusters or sub-clusters supports their distinctiveness as the basic species of edible Citrus.
15. However, the cpDNA sequence analysis of *rbcL* and *matK* gene could not find any clear cut differentiation between subgenera *Citrus* and *Papeda* as proposed in Swingle's system of classification.
16. This study was helpful in supporting the distinctiveness of *C. indica, C. latipes* and *C. ichangensis* as true species.
17. It also helped in elucidating the hybrid origin and relationships among the cultivated species/biotypes, such as *C. aurantiifolia, C. limon, C. limettioides, C. aurantium, C. sinensis, C. karna* and *C. macroptera.*
18. Seed storage behaviour of the species has been confirmed as intermediate exhibiting moderate seed longevity.
19. Seeds are desiccation and freezing sensitive which further confirm the need to conserve embryo or embryonic axes of this species using cryopreservation only.
20. Overall, among the three cryopreservation methods applied vitrification and encapsulation-dehydration werefound superior to air desiccation-followed by fast-freezing method in terms of higher recovery, while encapsulation resulted in slower growth of explants.
21. However, due to easy, cost effective and high reproducibility of technique air desiccation-followed by fast-freezing was recommended for long-term cryobanking citron genetic resources.
22. Cryopreservation was most efficient for the embryonic axes desiccated to 14-16% moisture content. Freezing of embryonic axes at 15.10% moisture content level in liquid nitrogen showed a good recovery rate of 70%, whereas,

embryonic axes possessing moisture contents at or above 20% and less than 9% lost viability completely when exposed to LN.

23. Successful base collection of Indian Citron germplasm and its utilization would be ensured using the studies conducted in this thesis.

ABSTRACT

Title: Genetic variability and Phylogenetic relationship studies in Indian Citron (*Citrus medica*. L)

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*Citrus medica*L. commonly known as Citronis considered as one of the three basic species of *Citrus.*Despite of various earlier systematic accounts,confusion still abounds in proper identification, taxonomic dispositionand circumscription of cultivated as well as wild and indigenousspecies/varieties of *Citrus* in India. In the present study, *C. medica* species was undertaken for investigation ofgenetic diversity and relationships of Indian citron with related genera.

Genetic diversity of forty six representative accessions of *Citrus medica* L. (Rutaceae) was estimated using 17 RAPD, 11 ISSR and 16 SSR markers.A total of 213 (RAPD), 105 (ISSR) and 66(SSR) bands were generated, out of which, 175 (RAPD), 77 (ISSR) and 52 (SSR) bands were polymorphic. Polymorphism percentage of RAPD (82%) was comparatively higher than ISSR (73%) and SSR (78.79%). Genetic similarity value was found in the range of 0.49 to 0.96 (average 0.73), 0.38 to 0.99 (average 0.69), 0.42 to 0.97 (average 0.70) and 0.49 to 0.96 (average 0.72) in RAPD, ISSR, SSR and combined data analyses, respectively. Moderately high levels of polymorphism and genetic similarity within *C. medica* suggested that citron cultivars have a significant level of genetic diversity. A dendrogram generated based on UPGMA separated all the accessions into two main clusters. The placement of *C. medica* accessions in various sub-clusters and groups in the dendrogram was based on molecular differentiation of individual accessions rather than their geographical origin. All the three markers i.e, RAPD, ISSR and SSR analyses were able to identify elite genotypes of *C. medica* with unique accession specific DNA fragments.

The taxonomy and molecular phylogeny in Indian Citron with other important *Citrus* species has been examined through sequence analysis of *rbcL* and *matK*gene region of chloroplast genome.The study was based on 23 accessions of *Citrus* genotypes representing 15 taxa of Indian *Citrus.* The phylogeny was inferred using the Maximum parsimony (MP) and Neighbor-Joining (NJ) methods. Both MP and NJ trees separated all the 23 accessions of Citrus into five distinct clusters. The chloroplast DNA (cpDNA) analysis based on *rbcL* and *matK* sequence data carried out in Indian taxa of *Citrus* was useful in differentiating all the true species and

species/varieties of probable hybrid origin in distinct clusters or groups. Sequence analysis based on *rbcL* and *matK*gene provided unambiguous identification and disposition of true species like *C. maxima, C. medica, C. reticulata* and related hybrids/cultivars.The separation of *C. maxima, C. medica* and C. *reticulata* in distinct clusters or sub-clusters supports their distinctiveness as the basic species of edible Citrus. However, the cpDNA sequence analysis of *rbcL* and *matK* gene could not find any clear cut differentiation between subgenera *Citrus* and *Papeda* as proposed in Swingle's system of classification.This study shows that the *rbcL* and *matK* sequence data can detect genetic variation in Indian *Citrus* genotypes, but the utility of the data in inferring phylogeny at intra and interspecific levels is limited probably by factors such as hybridization, bud mutations, apomixis and polyploidy.

Seeds of Indian citr*on*exhibited intermediate seed storage behaviour and had moderate seed longevity. The cryopreservation of embryonic axes of *C. medica*was attempted using air desiccation-freezing, vitrification and encapsulation-dehydration. Successful cryopreservation was achieved using all these three techniques.

सारांश

शीर्षक : भारतीय नींबू (*सिट्रस मेडिका* एल0) में आनुवांशिक विभिन्नता एवम् जातिवृत्तीय संबन्धों का अध्ययन

सिट्रस मेडिका एल0 को सामान्यतः सिट्रोन के रूप में माना जाता है जो कि सिट्रस की तीन बुनियादी प्रजातिओं में से एक है। पूर्व में दिए गए विभिन्न व्यवस्थित रिपोर्टो के बावजूद अभी भी भारत में नींबू के जंगली और देशी प्रजातिओं/किस्मों की उचित पहचान का ना होना, वर्गीकरण प्रकृति और सिमित पाबंदी के कारण इनकी अच्छी तरह से खेती करने के लिए भ्रम की स्थिति बनी हुई है। वर्तमान अध्ययन में *सी0 मेडिका* प्रजातिओ को उनकी आनुवांशिक विभिन्नताओं तथा भारतीय नींबू और उससे सम्बंधित पीढ़ीयों के साथ सम्बंधों की जाँच करने के लिए चुना गया।

सिट्रस मेडिका एल0 (रूटेसी) के 46 प्रतिनिधि एक्सेशनों में 17 आर ए पी डी, 11 आई एस एस आर तथा 16 एस एस आर मार्करों का उपयोग करके आनुवांशिक विभिन्नताओं का अनुमान लगाया गया जिनमे कुल 213 (आर. ए. पी. डी.), 105 (आई. एस एस आर) और 66 (एस एस आर) उत्पन्न हुए बैंड में से 175 (आर ए पी डी), 77 (आई एस एस आर) और 52 (एस एस आर) बैंड बहुरूपी थे। आर ए पी डी मार्कर की बहुरूपता प्रतिशत (82%), आई एस एस आर (73%) और एस एस आर (78.79%) मार्करो की तुलना में अपेक्षाकृत अधिक पायी गयी। आर ए पी डी, आई एस एस आर, एस एस आर और संयुक्त डेटा विश्लेषणों की आनुवांशिक समानता मूल्य क्रमशः 0.49 से 0.96 (औसत 0.73), 0.38 से 0.99 (औसत 0.69), 0.42 से 0.97 (औसत 0.70) और 0.49 से 0.96 (औसत 0.72) की रेंज में पाई गयी। *सिट्रस मेडिका* की आपस में उच्च स्तरीय बहुरूपता तथा आनुवांशिक समानता ने सुझाया की सिट्रोन कल्टीवर्स में मत्वपूर्ण स्तर की आनुवांशिक विविधता पाई जाती है। यू पी जी एम ए के आधार पर उत्पन्न डेड्रोग्राम ने सभी एक्सेशनों को दो मुख्य समुहो में बाँटा। *सि0 मेडिका* में एक्सेशनों को डेड्रोग्राम में उनकी भौगोलिक उदग्म के बजाय व्यक्तिगत एक्सेशनों को आणविक भेदभाव के आधार पर विभिन्न समूहो एवं उप–समूहों में स्थान दिया गया। सभी तीनों विश्लेषित मार्कर जैसे आए ए पी डी, आई एस एस आर, एस एस आर अद्वितीय परिग्रहण विशिष्ट डी एन ए, के टुकड़े के साथ *सिट्रस मेडिका* के सम्भ्रांत जीनोटाईपों की पहचान करने में सक्षम थे।

क्लोरोप्लास्ट जीनोम के 'आरबीसीएल' तथा 'मेटके' जीन क्षेत्र के अनुक्रम विश्लेषण के माध्यम से भारतीय नींबू का अन्य महत्वपूर्ण खट्टी प्रजातियों के साथ वर्गीकरण तथा आणविक जातिवृति की जाँच की गयी। यह अध्ययन भारतीय नींबू के पन्द्रह टेक्साओं का प्रतिनिधित्व करने वाले 23 सिट्रस जीनोटाइपों के एक्सेशनों पर आधारित था। इस जातिवृत अध्ययन का अनुमान अधिकतम मितव्यवता तथा नेबर–ज्योनिंग तरीको का उपयोग कर लगाया गया। इन दोनों अधिकतम मितव्यवता तथा नेबर ज्योनिंग वृक्षों के द्वारा नींबू में सभी 23 एक्सेशनों को 5 अलग–अलग समूहों में बांटा गया। नींबू के भारतीय टेक्सा में किए गए क्लोारोप्लास्ट डी एन ए विश्लेषण जो कि आरबीसीएल तथा मेटके अनुक्रम डेटा के आधार पर अंजाम दिया गया था जो सिट्रस की सभी सच प्रजातियों तथा प्रजातियों/किस्मों की संभावित संकर मूल को अलग समूहों में अंतर करने में उपयोगी था। आरबीसीएल और मैटके जीन के आधार पर अनुक्रम विश्लेषण, नींबू की सच प्रजातियाँ जैसे *सि0 मैक्सिमा, सि0 मेडिका, सि0 रेटिकुलेटा* और संबंधित संकर/कल्टीवर्स की स्पष्ट पहचान तथा उनके स्वभाव को दर्शाता है। अलग समूहों तथा उप–समूहों में *सि0 मैक्सिमा, सि0 मेडिका, सि0 रेटिकुलेटा* का विभाजन उनकी खाद्य नींबू की बुनियादी प्रजाति के रूप में विशिष्ठता का समर्थन करता है। हालांकि, आर बी सी एल तथा मैटके जीन के क्लोरोप्लास्ट डी एन ए अनुक्रम विशलेषण से स्विंगल के वर्गीकरण प्रणाली में प्रस्तावित उपवंश सिट्रस तथा पपेड़ा समूह के बीच कोई स्पष्ठ भेदभाव नहीं मिल सका है। इस अध्ययन से पता चलता है कि आर बी सी एल तथा मैटके अनुक्रम डेटा से भारतीय नींबू जीनोटाईपों में आनुवांशिक परिवर्तन का पता लगा सकते है। लेकिन संकरण, कली उत्परिवर्तन, एपोमिक्सिस और बहुगुणिता जैसे कारको से अतंर तथा अंतःप्रजाति स्तर की जातिवृत गणना की उपयोगिता का अनुमान लगाना शायद सिमित है।

भारतीय नींबू के बीजों ने मध्यवर्ती बीज भंडारण व्यवहार तथा मध्यम बीज दीर्धायु का प्रदर्शन किया। *सिट्रस मेडिका* के भ्रूणीय अक्षों का एयर डेसीकेशन, विट्रीफिकेशन तथा एनकेप्सुलेशन डीहाइड्रेशन तकनीकों द्वारा हिमपरिरक्षण का प्रयास किया गया। इन तीनों तकनीकों का उपयोग कर हिमपरिरक्षण में सफलता प्राप्त हुयी।

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APPENDIX – I

SOLUTION CHEMICALS AND REAGENTS USED FOR DNA EXTRACTION

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1. Liquid Nitrogen

2. Cetyl Trimethyl Ammonium Bromide (C-TAB 10%)

10g C-TAB was dissolved in 60 ml of sterile distilled water and kept for 15 min. at 60˚C in water bath and finally volume was made up to 100 ml with sterile distilled water and autoclaved.

3. Sodium Chloride (Nacl, 4M)

292.2 g of Nacl was dissolved in H_2O and volume was made up to 100 ml. The solution was autoclaved prior to use.

4. Tris HCL buffer (pH 8.0, 1M)

12.11 g of Tris salt was dissolved in distilled H_2O and volume was made up to 100ml and pH was adjusted to 8.0 using 1N HCL. The solution was autoclaved prior to use.

5. Ethylene Diamine Tetra Acetic acid (EDTA, 0.5 M)

18.62 g EDTA was dissolved in sterile distilled H_2O. The pH of the solution was adjusted to 8.0 using 1 B NaOH. The volume was made up to 100 ml using sterile distilled H_2O and the solution was autoclaved.

6. β- Mercaptoethanol 2 percent solution

2 percent solution provided by the manufacturer was stored in an amber coloured bottle.

7. DNA extraction buffer

DNA extraction buffer was prepared afresh as and when necessary using stock solutions of the individual components.

Extraction Buffer composition

Component	Stock solution	Working buffer	Vol. of stock taken to prepare 200 ml buffer
C-TAB	10%	1.5%	40 ml
Nacl	4 M	1.4 M	70 ml
Tris	1 M	100 mM	20 ml
EDTA	0.5 M	20 mM	8 ml
B-Mercaptoethanol*	2%	2%	4 ml
Distilled H_2O	-	-	58ml

*Mercaptoethanol was added at last after adding extraction buffer to ground plant sample.

8. Isopropanol

9. Sodium Acetate 3M, pH 5.6

30.75 g sodium acetates was dissolved in sterile distilled H_2O, pH was adjusted to 5.6 with glacial acetic acid and volume made up to 50 ml. The solution was autoclaved and stored till use.

10. Chloroform: Isoamyl alcohol (24 : 1) mixture

96 ml of chloroform was mixed with 4 ml of isoamyl alcohol. It was stored in amber coloured bottle.

11. 70% Ethanol

70 ml of absolute ethanol was mixed well with 30 ml of sterile water and stored in a stopper bottle till use.

DNA PURIFICATION

1. **Phenol: Chloroform: Isoamyl alcohol (25:24:1) mixture.**

   100 ml of Tris saturated phenol was added to a mixture of 96 ml chloroform and 4 ml isoamyl alcohol. The mixture was mixed well prior to use and stored in amber coloured bottle.

2. **RNase (20 mg/ml) solution**

RNase	20mg
Tris-Cl (pH 7.5)	10 mM
NaCl	1.5 mM

   Sterile water was added to make the volume to 1 ml. The solution was heated at 100˚C for 15 minutes to inactivate any DNase presented then stored in aliquots at -20˚C.

SOLVENT FOR DNA

1. **Tris: EDTA (TE) buffer (10 mM Tris: 1 mM EDTA, pH 8.0)**

   10 ml of Tris (1M) Buffer, pH 8.0 and 0.2 ml of 0.5 M EDTA, pH 8.0 was mixed with sterilized H_2O and volume made up to 100 ml. The solution was autoclaved prior to use.

2. **10 X TAE buffer stock solution (100 mM Tris: 10 mM EDTA: 1M NaCl, pH 7.4)**

   12.11 g Tris, 3.72 g EDTA and 58.44 g of Nacl were dissolved in sterile distilled water and volume was made up to 1000 ml using distilled water. The pH was adjusted to 7.4 with HCL solution, filtered before use and stored at 4˚C.

GEL ELECTROPHORESIS

1. **Agarose gel (1.8%)**

   3.6 g agarose was made up to 200 ml with 1 X TAE buffer; the contents were mixed thoroughly for 3-4 min. to dissolve the contents. The mixture was cooled down to 40˚C. The molten was casted in a gel tray with a comb containing 30 teeth to produce the veil.

2. **Ethidium Bromide (10 mg ml^{-1})**

10 mg of ethidium bromide was dissolved in sterile distilled water and volume made upto 1 ml. The solution is then stored in amber colour bottle at 4˚C.

3. **Loading dye (10 X solution)**

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol	50%
TAE	1 X

Sterile water was added to the above compound to make volume to 100 ml.

PCR COCKTAIL

1. ***Taq* DNA Polymerase**

A stock solution of 3 units μl^{-1} was provided by the manufacturer (Bangalore Genei) was stored at -20˚C.

2. **Deoxyribonucleotide Triose Phosphates**

dATP (10 mM)	20 µl
dGTP (10 mM)	20 µl
dCTP (10 mM)	20 µl
dTTP (10 mM)	20 µl
Sterile water	20 µl

The solution was mixed well and stored at -20˚C till use.

3. **Magnesium chloride**

A solution of 25 mM μl^{-1} provided by the manufacturer was used. Storage was at -20˚C.

4. **Primer**

The primer was provided by the manufacturer in a lyophilized form. Based on the molecular weight of a given primer, a solution of 10 µM was prepared by adding the required amount of sterile water stored at -20˚C.

APPENDIX- II

CRYOPROTECTANT SOLUTIONS AND

Preparation of Preculture Medium

The pre-culture medium may contain 0.3 – 1 M of a dehydrating agent (mannitol, sucrose or sorbitol) in basal medium (semi-solid or liquid). The medium is dispensed in culture tubes enclosed with polypropylene caps and autoclaved for 15 min. The medium is cooled at room temperature and the time of use, approximately 10 ml of molten medium is poured in plastic Petriplates (60 mm diameter) and allowed to solidify.

Preparation of Vitrification Solution

Composition of Plant Vitrification solution 2 (PVS2)

The plant vitrification solution, referred as PVS2 solution (Sakai et al., 1990), contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose in MS medium. The pH is adjusted to 5.8. For preparing 100 ml of the solution, the volume of the respective components is as detailed below:

Glycerol 30%	28.6 ml
Ethylene Glycol 15%	14.8 ml
DMSO 15%	14.8 ml

Preparation of Plant Vitrification Solution 2 (PVS2)

a. In a 100 ml graduated cylinder add 30 ml of MS liquid medium containing 0.4 M sucrose (136.92 g/l) and a small stir bar.
b. Add 14.8 ml DMSO and stir well.
c. Add 14.8 ml Ethylene Glycol and stir well.
d. Slowly add 28.6 ml Glycerol while stirring.
e. Let stir until dissolved.
f. Make up the volume to 100 ml with MS liquid medium containing 0.4 M sucrose and adjust the pH to 5.8
g. Once prepared, PVS2 solution is autoclaved and stored for future use.

Preparation of unloading solution

To prepare the above, 1.2 M sucrose (41.0 g/100 ml) is dissolved in liquid MS medium and volume made up to 100 ml. Following adjustment of pH to 5.8, the solution is autoclaved and stored for future use.

Preparation of Encapsulation Solutions

Sodium alginate solution (3%)

a) For preparing 100 ml of alginate solution, add required stock solutions of MS medium (except halides) (Table) along with sucrose (0.08-0.4 M).
b) Make up the volume to 100 ml and adjust pH to 5.8. Transfer the contents to a conical flask.
c) Keeps the above flask containing MS stocks on a medium heat on a hot plate cum magnetic stirrer.
d) Weigh 3.0 gm sodium alginate (low viscosity, usually Sigma A 2158) and add it little by little to the flask containing MS stock while stirring it constantly with a glass rod.
e) Continue adding and stirring sodium alginate till it goes into solution. Take care to break clumps of sodium alginate using the glass rod.
f) Autoclave the alginate solution and store it for use.

Calcium chloride solution

To prepare the above, 100 mM (7.3 g/500ml) calcium chloride is dissolved in liquid MS medium and volume made up to 500 ml. Following adjustment of pH to 5.8, the solution is dispensed into smaller flasks (100 ml each), autoclaved and stored for future use.

Sucrose dehydration solution

To prepare the above, 0.75 M sucrose (25.6/100 ml) is dissolved in liquid MS medium and volume made up to 100 ml. Following adjustment of pH to 5.8, the solution is autoclaved and stored for future use.

Table: Constituents for Stock Solution Preparation for Murashige & Skoog (MS) medium

Chemicals	Conc. of stock solution (×100)	Amount of stock per litre of medium
A.	(1000ml)	
Phosphate borate molybdate	17.0 g	10 ml
KH_2PO_4	0.62 g	
H_3BO_3	25 mg	
$Na_2MoO_4.2H_2O$		
B.	(1000ml)	
Sulphate		
$MgSO_4.7H_2O$	37.0 g	10 ml
$MnSo_4.4H_2O$	1.69 g	
$ZnSo_4.7H_2O$	0.86 g	
$CuSo_4.5H_2O$	2.5 mg	
C. Halides	(1000 ml)	
$CaCl_2.2H_2O$	44.0	
KI	83.0	10 ml
$CoCl_2.6H_2O$	2.5 mg	
D. Nitrate	(1000 ml)	
NH_4NO_3	165.0 g	10 ml
KNO_3	190.0 g	
E. Iron	(1000 ml)	
Na_2 EDTA.$2H_2O$	3.73 g	10 ml
$FeSo_4.7H_2O$	2.78 g	
F. Vitamins	(100 ml)	
Pyrodoxine.HCL	50.0 mg	
Nicotinic acid	50.0 mg	1 ml
Thiamine.HCL	20.0 mg	
Glycine	200 mg	

G. Growth regulators	(10 ml each)	
NAA	10 mg	Quantity may vary depending on the experiment
BAP	10 mg	
2 iP	10 mg	
Zeatin	10 mg	

Note: All the constituents were dissolved in 250 ml distilled water separately and volume was made upto 1000 ml. 10 ml of stock was used per litre of the medium.