Chapter 2

REVIEW OF LITERATURE
The genus, *Ziziphus* (jujube), a member of Rhamnaceae is comprised of approximately 170 species of deciduous/evergreen trees and shrubs distributed in the tropical and subtropical regions of the world (Liu and Cheng, 1995) between 34°S and 51°N latitude, and up to 2800 m above sea level in different continents (Table 1) with a few species occurring in temperate regions. The number of species reported in the genus *Ziziphus* is variable: 18 species and 5 intermediate species (Hooker, 1875), 50 species (Watt, 1893), 60 species (Ridley, 1922), 40 species (Rendley, 1959), 80 species (Evreinoff, 1964), 86 species (Johnston, 1972), 135 species of which 90 are found in the old world and 45 in the new world (Bhansali, 1975) and 170 species (Liu and Chang, 1995). Out of the total species in the genus *Ziziphus* 88 (51.8%) are concentrated in Asia, followed by North America (31), South America (28), Africa (28), Oceana (9) and Europe (5). However, the most distinctive species are found in North and South America (28 and 27, respectively) (Liu and Cheng, 1995). Some species, like *Z. mauritiana* and *Z. jujuba*, occur nearly in every continent, whereas other species, like *Z. nummularia*, *Z. spin-a-christi* and *Z. mucronata* are restricted to distinct areas (Table 2.1). *Ziziphus* species can grow either as trees and shrubs (*Z. mauritiana*, *Z. rotundifolia*, *Z. jujuba*, *Z. mucronata*) or exclusively as small shrubs or bushes (*Z. nummularia*, *Z. lotus*, *Z. spin-a-christi*, *Z. obtusifolia*).

In both China and India, *Ziziphus* trees have a long tradition of selection and cultivation, with the result that the species occurring in these countries (*Z. mauritiana*, *Z. jujuba*) are better known and more widely researched than those in other regions. These two species are economically important and being cultivated for their fruit (Islam and Simmons, 2006). *Ziziphus mauritiana* Lam, also known as ber/Indian jujube/Indian
plum/ Chinee Apple / Desert apple is an evergreen, medium-sized, thorny tree with ability to thrive and produce fruits in arid or semi-arid regions. It is native from the Province of Yunnan in southern China to Afghanistan, Malaysia and Queensland, Australia. It is cultivated to some extent throughout its natural range but mostly in India where it is grown commercially and has received much horticultural attention and refinement despite the fact that it frequently escapes and becomes a pest.

Table 1: Worldwide Distribution of *Ziziphus* Species

<table>
<thead>
<tr>
<th>Continent</th>
<th><em>Ziziphus</em> species</th>
<th>Part of continent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa</td>
<td><em>Z. abyssinica</em> Hochst.</td>
<td>Tropical Africa</td>
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<tr>
<td></td>
<td><em>Z. lotus</em> Lamk.</td>
<td>Northern Africa</td>
</tr>
<tr>
<td></td>
<td><em>Z. mauritiana</em> Lamk.</td>
<td>Tropical Africa, Sahel Zone, Zimbabwe</td>
</tr>
<tr>
<td></td>
<td><em>Z. mucronata</em> Willd.</td>
<td>Southern Africa</td>
</tr>
<tr>
<td></td>
<td><em>Z. spina-christi</em> Willd.</td>
<td>Middle east</td>
</tr>
<tr>
<td>Asia</td>
<td><em>Z. jujuba</em> Mill.</td>
<td>China, India, Korea, Malaysia</td>
</tr>
<tr>
<td></td>
<td><em>Z. mauritiana</em> Lamk.</td>
<td>China, India, Pakistan, Malaysia</td>
</tr>
<tr>
<td></td>
<td><em>Z. nummularia</em> W.i.A.</td>
<td>India</td>
</tr>
<tr>
<td></td>
<td><em>Z. oenoplia</em> Mill.</td>
<td>Tropical Asia</td>
</tr>
<tr>
<td></td>
<td><em>Z. rotundifolia</em> Lam.</td>
<td>India</td>
</tr>
<tr>
<td></td>
<td><em>Z. rugosa</em> Lam.</td>
<td>India</td>
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<tr>
<td></td>
<td><em>Z. sativa</em> Gaertn.</td>
<td>Pakistan</td>
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<tr>
<td></td>
<td><em>Z. spina-christi</em> Willd.</td>
<td>Middle east</td>
</tr>
<tr>
<td></td>
<td><em>Z. xylopyra</em> Willd.</td>
<td>India</td>
</tr>
<tr>
<td>Australia</td>
<td><em>Z. mauritiana</em> Lamk.</td>
<td></td>
</tr>
<tr>
<td>Europe</td>
<td><em>Z. jujuba</em> Mill.</td>
<td>Mediterranean</td>
</tr>
<tr>
<td></td>
<td><em>Z. lotus</em> Lamk.</td>
<td>Mediterranean</td>
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<td></td>
<td><em>Z. mauritiana</em> Lamk.</td>
<td>Mediterranean</td>
</tr>
<tr>
<td></td>
<td><em>Z. sativa</em> Gaertn.</td>
<td>Mediterranean</td>
</tr>
<tr>
<td>North America</td>
<td><em>Z. amole</em> M.C.Johnst.</td>
<td>Mexico</td>
</tr>
<tr>
<td></td>
<td><em>Z. celata</em> J.i.H.</td>
<td>USA</td>
</tr>
<tr>
<td></td>
<td><em>Z. jujuba</em> Mill.</td>
<td>USA</td>
</tr>
<tr>
<td></td>
<td><em>Z. mexicana</em> Rose</td>
<td>Mexico</td>
</tr>
<tr>
<td></td>
<td><em>Z. obtusifolia</em> Gray</td>
<td>Mexico, USA</td>
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<tr>
<td>South America</td>
<td><em>Z. cinnamomeum</em> Tr.&amp;Pl.</td>
<td>Venezuela</td>
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<td></td>
<td><em>Z. mistol</em> Griseb.</td>
<td>Argentinia, Paraguay</td>
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<tr>
<td></td>
<td><em>Z. joazeiro</em> Mart.</td>
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<td></td>
<td><em>Z. oblongifolia</em> S.Moore</td>
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The tree does best on sandy loam, neutral or slightly alkaline. It also grows well on laterite, medium black soils with good drainage, or sandy, gravelly, alluvial soil of dry riverbeds where it is vigorously spontaneous. These trees perform well even on marginal and inferior lands where most other fruit tree species either fail to grow or give poor performance (Jawanda and Bal, 1978). In India during summer months of May and June, *Z. mauritiana* enters into dormancy by shedding its leaves. The scions of varieties which have been selected to improve the yield and fruit quality are routinely grafted on to the vigorous rootstocks of wild species to provide a reasonable cash crop on land which is unsuitable for other forms of cultivation (Cherry, 1985). *Ziziphus* species have several physiological and morphological characteristics that may contribute to their ability to adapt to arid environments and make it important for the integrated economy of the arid lands. Species of fruit trees in the genus *Ziziphus* represent examples of such multipurpose plants with great potential for selection and use in drought-prone regions. *Z. mauritiana* is an example of an extremely drought-hardy species, and is a dominant component of the natural vegetation of the Indian desert (Cherry, 1985). These trees are well adapted to seasonal drought and hot conditions. They play an important role in the soil erosion with their strong root system that ensures its ability to exploit deep water sources, thereby maintaining a sufficient water and nutrient supply for prolonged periods when the upper soil layers are drying out (Singh *et al.*, 1998) and assimilation/leaf conductance ratio than in unstressed trees (Jones, 1992). An indication of the importance of the root is the high root-to-shoot ratio of *Z. mauritiana* and deep rooting which has been reported as a characteristic of both *Z. nummularia* and *Z. mauritiana* (Depommier,
1988). The leaves of *Ziziphus* provide fodder for livestock; the hard wood is used for making agricultural implements, fuel and high quality charcoal. In many regions, *Ziziphus* is grown as a hedge, with its spines creating effective live fencing. Its fruits are highly nutritious and provide a valuable source of energy, vitamins. In addition, extracts from fruits, seeds, leaves, roots and bark of the trees are used in many traditional medicines to alleviate the effects of insomnia, skin diseases, inflammatory conditions and fever. Juice of the root bark is said to alleviate gout and rheumatism and an infusion of the flowers serves as an eye lotion. *Ber* trees appear to tolerate roadside pollution because they maintain high chlorophyll content, particularly chlorophyll b, although their growth becomes retarded (Hussain *et al.*, 1994). Plants affected by fire damage regenerate in short time. The regrowth is mainly from new shoots that arise from just above or just below ground level in the case of young plants and from the buds in the canopy or epicormic buds on the stems of older plants (Grice and Brown, 1996; Grice, 1997).

At about the beginning of the Christian era, the Chinese jujube was imported into Europe and is now widely distributed throughout Persia, Armenia, Syria and the Mediterranean regions in Spain and France. Reports of early writers and explorers emphasized the heat and drought tolerance of jujubes, and probably because of this, jujubes were thought most likely to succeed in the dry regions of the southwestern US (Locke, 1947). *Z. mauritaina* was introduced into Guam about 1850 but is not often planted there or in Hawaii except as an ornamental. Specimens are scattered about the drier parts of the West Indies, the Bahamas, Colombia and Venezuela, Guatemala, Belize, and southern Florida. In Barbados, Jamaica and Puerto Rico the tree is naturalized
and forms thickets in uncultivated areas. In 1939, six trees from Malaysia were introduced into Israel and flourished there. Some improved Indian cultivars like Gola and Seb have been imported to Israel and Africa and are the grafts onto native rootstocks of Z. spina-christi and Z. abyssinica, respectively (Cherfas, 1989). The same technique was successfully used in Zimbabwe to produce high-quality Indian selections on the native Z. nummularia rootstock species (Kadzere and Jackson, 1997).

The fruit of ber is known as drupe. The fleshy drupes of several species are rich in sugars and vitamins, and this fact has made. The skin of fruit most is smooth and greenish-yellow to yellow. The drupes are eaten fresh, pickled, dried or made into confectionery, and the juice can be made into a refreshing drink (Khoshoo and Subrahmanyam, 1985). In Zimbabwe, Ziziphus fruits are used to produce jam and kachaso, a crudely distilled spirit of consirable potency (Coates Palgrave, 1990). Though the fruit does not find much favour with the upper classes (poor man’s fruit, Khoshoo and Subrahmanyam, 1985), it has a high nutritional value and a great commercial potential. Consequently, in many regions of the world, Ziziphus fruits are sold on local markets, generating cash income for people of rural areas and improving family nutrition. Drupes have higher contents of protein and vitamins A and C than apples (Anonymous 1976). The fruits contain between 70 and 165 mg ascorbic acid per 100 g of pulp, which is two to four times higher than the vitamin C content of citrus fruits. The mineral content of calcium, phosphorus and iron in Z. mauritiana fruits is also reported as being higher than in apples and even oranges (Jawanda and Bal, 1978). For farmers, Z. mauritiana is an interesting crop because it is so fast growing and bears fruits within 2-3 years (Jawanda
and Bal, 1978). Jujubes (Z. jujuba) were eaten by the ancients of the chalcolitic age (1500-1000 b.c.) and the fruits have been in cultivation for the past 400 years in both India and China (Anonymous, 1976).

The food value per 100 gm of edible portion of fresh fruit includes: moisture (81.6-83.0 g), protein (0.8 g), fat (0.07 g), fiber (0.60 g), carbohydrates (17.0 g), total sugars (5.4-10.5 g), reducing sugar (1.4-6.2 g), non-reducing sugar (3.2-8.0 g), ash (0.3-0.59 g), calcium (25.6 mg), phosphorous (26.8 mg), Iron (0.76-1.8 mg), carotene (0.021 mg), thiamine (0.02-0.024 mg), riboflavin (0.02-0.038 mg), niacin (0.7-0.873 mg), citric acid (0.2-1.1 mg), ascorbic acid (65.8-76.0 mg), fluoride (0.1-0.2 ppm) and pectin (2.2-3.4%).

Under ideal environmental conditions, Z. mauritiana exhibits very high rates of net photosynthesis and stomatal conductance, high rate of nitrate reductase activity (NRA) in leaves of Z. mauritiana (1 µmol NO₂⁻ g FM⁻¹ h⁻¹) measured in leaves of drought-stressed plants. Consequently, total nitrogen content of the leaves is very high, which is a mesic character of Ziziphus leaves, that lack xeromorphic adaptations such as heavy cuticularization, or deep folds in their surfaces with sunken stomata. The combination of high levels of NRA and net photosynthesis results in a high relative growth rate, essential if these plants are to compete effectively during brief periods of active growth. The large carbohydrate reserves in the roots contribute to the strong regeneration potential of Ziziphus plants. Z. mauritiana is reported as having a great power to recover from injury of any kind, including fire, and thrives on burnt grassy tracts (Anonymous, 1976; Grice, 1996). After such events, most plants of Z. mauritiana
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resprouted vigorously within 3 months and by the 4th month after fire, burnt and unburnt plants were similar with respect to the distribution of individuals and their physiological characteristics (Grice, 1997). Because of its ability to resprout from both crown and roots, along with its resistance to herbicides, Z. obtusifolia has demonstrated its ability to increase its cover on Texas rangeland after the release of competition from other woody vegetation by bush control treatments (Speer and Wright, 1981). There are reports that wild jujube plants (Z. lotus) also have the ability to resprout vigorously even after being cut to ground level in the previous fall in Morocco (Regehr and El Brahli, 1995). Ziziphus plants are cross-pollinated and are highly out breeding, and as a result of this, the natural population, which largely regenerates through seeds, exhibits a vast range of genetic heterogeneity. The potential of this variability has been severely underutilized. In contrast, the commercial cultivars, which are clonally propagated via budding on a suitable rootstock, have retained their genetic fidelity. In evolutionary terms, this genetic variability may well benefit Ziziphus in harsh environments by allowing rapid adaptation to changing environmental conditions at a population level.

In India, more than 90 cultivars differing in the habit of the tree, leaf shape, fruit form, size, color, flavor, keeping quality, and fruiting season have been reported. Among the important cultivars, eleven are described in the encyclopaedic Wealth of India: 'Banarasi (or Banarsi) Pewandi', 'Dandan', 'Kaithli' ('Patham'), 'Mundia Mahrara', 'Narikelee', 'Nazuk', 'Sanauri 1', 'Sanauri 5', 'Thornless' and 'Umran' ('Umri'). ber cultivation has not assumed large proportion due to several constraints. ber orchards are
located mainly in ecologically deprived, stress prone areas where production, marketing and managerial skills are poorly developed. The farmers face the following constraints:

A) Unavailable information on (i) Modern production techniques, and (ii) Post harvest management and marketing

B) Lack of inputs for (i) reliable planting material of recommended variety, and (ii) reliable plant protection equipments and chemicals

C) Absence of infrastructures for (i) regulatory mechanism to ensure supply of good quality and reliable planting material, and (ii) checks to regulate supply of good quality plant protection chemicals.

Research initiatives on ber in India until the 1960 were limited to varietal trials and vegetative propagation studied in the states of Punjab and Uttar Pradesh. Major research initiatives took place during the last four decades of the twentieth century.

2.2 Molecular Markers and Their Use in Plants

Plant genetic resources are one of the most valuable assets available to mankind. Protection, preservation and conservation of these resources for posterity, therefore, assume enormous significance. An important component for effective and efficient management of plant genetic resources as well as their utilization is characterization of the germplasm. Such a characterization is essential not only for identification of various species but also to determine their genetic relatedness. The information generated could be used successfully in breeding programmes wherever possible. This also assumes great relevance in the present context of intellectual property rights and trade agreements.
Markers for identifying and manipulating genes have been in use since decades (Tanskley, 1983). Being heritable entities, their association with economically important traits can be potential selection tools in crop breeding research. Broadly they may be classified into morphological, biochemical and molecular markers. Assessment of diversity has traditionally been through morphological characters, which has often found to be rather less effective.

2.3 Morphological Markers

Morphological markers are observable and that segregate with the trait(s) of interest. Sax (1923) first reported association of a simple inherited genetic marker with the quantitative trait in plants when he observed segregation for seed size associated with segregation of seed coat colour in beans. Plant breeders and geneticists extensively studied and employed the linkage between morphological markers and the economically important traits, which in most cases are quantitative in nature. Prior to the advent of isozymes and RFLP as markers, the major technique of gene location was the use of chromosome translocation. (Kang et al., 1979) Once the location of major gene on a specific chromosome has been established, information on its linkages with flanking markers can be obtained via three-point cross linkage analysis.

Morphological traits or markers are, however, very limited in number, environmentally influenced, stage specific expression, relative expressivity and penetrance, pleiotropy etc. Therefore, there was urgent need for more efficient marker system, possibly covering the whole genome without the limitations.
2.4 Biochemical Markers

Biochemical markers reveal polymorphism at protein level (Scandalios, 1969). In the 1980's, isozymes were first used as a biochemical marker system and used extensively to map some important traits. These markers have been used to identify QTL in maize, tomato, wild oats and soybeans (Stuber, 1992), variation in Korean jujube (Oh et al., 1988). These markers gained greater success than the morphological markers. It eliminated the need for having gene markers with discrete and visible effects of the phenotype. However, isozyme analysis has its inherent disadvantages like limited number of enzyme loci, vulnerability to environmental influence and post-translation modifications and stage specific expression. Flavanoid pattern in ber has been studied to differentiate among genotypes by Vashishtha et al. (1989)

2.5 Molecular Markers

With the advent of molecular biological technique, DNA based markers have replaced enzyme markers in germplasm identification and characterization. DNA markers have distinct advantages over other markers as they are plastic, ubiquitous, stable and unlimited in number, discrete and non-deleterious, inherited in simple Mendelian fashion, covering the whole genome and have none of the problems experienced with morphological markers and free from epistatic and interaction and pleiotropic effects (Swathi et al., 1999). The DNA markers are more widely employed for molecular tagging of genes and molecular marker assisted selection (Gupta et al., 1999). In several crops, molecular markers closely linked to numerous traits of
economic importance have been developed (Caetano-Anolles and Gresshoff, 1997) that allows indirect selection for desirable traits in early segregating generations at the seedling stage by side stepping the confounding effect of the environment. Today various types of DNA markers are employed for identification and manipulation of genes governing agronomically important traits in many crop plants. Ideal DNA markers should have some desirable properties such as: Highly polymorphic nature, codominant inheritance (determination of homozygous and heterozygous states of diploid organisms), frequent occurrence in genome, selective neutral behaviour (the DNA sequences of any organism are neutral to environmental conditions or management practices), easy access (availability), easy and fast assay, high reproducibility and easy exchange of data between laboratories.

The DNA markers systems have also been classified as the first second and third generation molecular marker systems. The first generation markers are mainly the RFLP and RAPD and their modifications, while the second generation molecular marker systems include AFLP, STMS and their modifications. Third generation molecular marker system are expressed sequence tags (Est’s) and the Single Nucleotide Polymorphisms (SNPs). DNA markers may be broadly classified into two types (i) Hybridization based markers and (ii) PCR based markers.
2.6 Hybridization Based Markers

These markers involve DNA: DNA hybridization, which in turn depends on base pairing rule. RFLP and VNTR fall under this category. Even though these markers are highly informative but require laborious procedures and use of hazardous radioisotopes.

2.6.1 Restriction fragment length polymorphism (RFLP)

It is one of the first marker systems developed and the technique is basically dependent on two steps viz., restriction digestion and detection of specific fragment(s) using specific probe, Restriction endonucleases cleave total genomic DNA at specific sites, the digested DNA is then separated on Agarose gel and blotted to membrane and hybridized with radiolabeled specific probe, which detects specific homologous fragments. The membrane is then washed to remove the un-hybridized probes and subsequent detection of fragments by autoradiography (Botstein et al. 1980). RFLPs promise to be highly reproducible, codominant in nature, allowing differentiation of heterozygotes from homozygotes, multiple allelic forms and absence of pleiotropic effects on economic traits (Beckmann and Soller, 1983). Polymorphism detected by RFLP is basically due to the differences in position of restriction sites. Though its advantage on, it is time consuming technique, requires the use of radioactive isotopes and it has generally low level of polymorphism compared to the other PCR based marker systems. It also requires high quantities of good quality DNA and where only very limited amounts of source material are available, this requirement alone may
preclude its application. The use of single copy probes gives a simple banding pattern, which could be used easily for mapping and tagging genes of economic importance. A deviation from the basic RFLP is the use of multi-locus probes, which produce a complex pattern, and is ideal for DNA fingerprinting and individual identification. Two categories of such multi-locus probes are mainly used, *Minisatellites*, which is a tandem repeats of a basic motive of 10-60 bp (Jeffreys *et al.*., 1985) and *microsatellite*, which is repeats of a basic motive of 1-6 bp (Ali *et al.*., 1986).

Ragot and Hoisington (1993) compared the time efficiency and cost of RFLP (chemiluminiscent and radioactivity-based) and RAPD from simulation of maize genotyping experiments. The increase in total cost with increasing number of individuals genotyped and markers analyzed is higher for RAPD than for RFLPs. RAPDs generally were found to be more costly and time efficient for small sample size while RFLPs were suitable for bigger sample size. In RFLPs, cRFLP (chemiluminiscent) requires less exposure time than rRFLP (radioactivity-based) to obtain a given amount of information.

2.6.2 Variable number tandem repeat (VNTR)

The term VNTR refers to the variable sequence rather than to the method used to detect it (Nakamura *et al.*., 1987). These sequences are scattered at various locations in the human genome regions that are highly variable. Polymorphisms appear because of variation in the number of tandem repeats (VNTR loci) in a given repeat motif. Tandem repeats are multiple copies of a sequence of base pairs arranged in head to tail fashion. For example, a frequently found tandem repeat is CA, and one strand containing this type
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of repeat reads CACACA, notated as (CA)n. The other strand would read GTGTGT… In
this example, the number of repeating base pairs is two, but it can be more. When the
repeating unit is less than four, the VNTR is called a microsatellite and when the
repeating unit is longer it is a minisatellite. Short identical segments of DNA aligned
head to tail in a repeating fashion are interspersed in the human genome. Some VNTR
sequence segments are found at only a single locus in the human genome. Probes made of
these sequences are single locus probes and yield patterns such as those at left when used
to probe RFLP blots of DNAs of six individuals.

2.7 PCR Based Markers

The in vitro amplification of DNA by the polymerase chain reaction (PCR)
(Saiki et al., 1985) has proven to be revolutionary technique in molecular biology. It
facilitates in-vitro amplification of DNA by using two oligonucleotide primers
complementary to opposing DNA strands. PCR is a rapid inexpensive and technically
simple. PCR-based approaches are in demand because of their simplicity and
requirement for only small quantities of sample DNA. By combining a thermostable
DNA polymerase with repeated cycling through reaction conditions favoring DNA
template denaturation, followed by template-primer annealing and finally primer
extension, exponential DNA amplification occurs. Beginning with as little as one
molecule of DNA, microgram quantities of a specific DNA fragment is produced in a
couple of hours. Because of these features a multitude of new genotyping methods. The
PCR based methods can be divided into two categories: (i) sequence-arbitrary methods,
and (ii) those requiring a priori sequence information (Reiter, 2001).
2.7.1 Sequence-arbitrary primers

Because in many plant species (a) the need of availability of large amount of sequence information is limited in many species and obtaining additional sequence information can be cost-prohibitive, (b) based upon available sequence, the degree of polymorphism revealed by PCR amplification and fragment size separation is low, the development and use of sequence-arbitrary methods has occurred primarily with plant species. This include Random Amplified Polymorphic DNA (RAPD), Arbitrary Primed PCR (AP-PCR), DNA Amplification Fingerprinting (DAF), Inter Simple Sequence Repeat (ISSR) and Amplified Fragments Length Polymorphism (AFLP). These methods allow simultaneous assaying of 3-30 genomic sites. One drawback, however of these techniques is that the primers used are usually AT rich that amplify the heterochromatin region (non-genic region) more frequently. Moreover, annealing temperature is usually very low (37\(^0\)-40\(^0\)C) which provides for non-specific annealing to some extent and hence amplification may not be reproducible. AFLP and ISSR overcome this limitation by using highly stringent annealing conditions.

2.7.1.1 Random amplified polymorphic DNA (RAPD)

Amplification of genomic DNA, using single and short primer (10bp), under low stringency conditions (low annealing temperature due to the low primer Tm value \(i.e.\ 35-37^0C\)), results in multiple amplification products distributed randomly throughout the genome. This is called Random Amplified Polymorphic DNA (RAPD), (Williams \textit{et al.}, 1990). This technique is widely accepted due to its simplicity and used extensively
for DNA fingerprinting, genome mapping (Williams et al., 1990), gene tagging (Martin et al., 1991, Paran et al., 1991), population and polygenetic studies (Van Heusden and Bachmann, 1992) and varietal identification (Farooq et al., 1995; Devanshi et al., 2007) and genetic diversity analysis (Zhang et al., 2005) in different food and fruit crops. There are a number of advantages of RAPD over the RFLP namely; simplicity, lower cost/sample, non-use of radioactivity and the higher level of polymorphism. On the other hand, reproducibility of RAPD has been a subject of considerable discussion as it generally has low reproducibility.

**Principle of RAPD markers**

The principle involved in generating RAPDs is that, a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that the amplified fragments generated by PCR depend on the length and size of both the primer and the target genome. The assumption is made that a given DNA sequence (complementary to that of the primer) will occur in the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR. These amplified products (of up to 3.0 kb) are usually separated on agarose gels and visualized by ethidium bromide staining. The use of a single 10-mer oligonucleotide promotes the generation of several discrete DNA products and these are considered to originate from different genetic loci. Polymorphisms result from mutations or rearrangements either at or between the primer binding sites and are detected as the presence or absence of a particular RAPD band. This means that RAPDs are dominant markers and therefore cannot be used to identify
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heterozygotes. Several factors have been shown to affect the number, size and intensity of bands. These include PCR buffers, dNTPs, Mg\textsuperscript{2+} concentration, cycling parameters, source of Taq polymerase, condition and concentration of DNA and primer concentration. Results obtained by RAPDs are highly prone to user error and bands obtained can vary considerably between different runs of the same sample. This limitation is, however, easy to overcome through practice and many articles have reported consistency in the profiles obtained from different runs of the same sample (Lerceteau et al., 1997; Clerc et al., 1998). The problem of the reproducibility of RAPDs data between laboratories that use different PCR machines may soon disappear with the availability of new improved PCR machines on the market. Maier et al. (1994) have reported identical banding patterns obtained using different thermocyclers and success depends mainly on a defined set of conditions, which should be maintained constant when using different machines.

Impact of scoring error and reproducibility of RAPD data was estimated by Skroch and Nienhuis (1995) and they reported the probability of a scored RAPD band being scored in replicate data is strongly dependent on the uniformity of amplification conditions between experiments, as well as the relative amplification strength of the RAPD band. They observed significant improvement in the reproducibility of scored bands and some reduction in scoring error can be achieved by reducing differences in reaction conditions between replicates.

Other related techniques include Arbitrary Primed PCR (AP-PCR) (Welsh and McClelland, 1990) and DNA Amplification Fingerprinting (DAF) (Caetano-Annoles et
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al., 1991). These methods differ from RAPDs in primer length, the stringency conditions and the method of separation and detection of the fragments.

2.7.1.2 Arbitrary primed PCR (AP-PCR)

Arbitrary Primed PCR (AP-PCR) provides plus/minus DNA amplification based polymorphisms. In this technique, longer single primer is used (22-24bp) and primer concentrations are 10-fold higher (Welsh and McClelland, 1990). The thermal cycling profile begins with one or two cycles incorporating a low annealing temperature (40-48°C typical) followed by 30 cycles using a high stringent annealing temperature and radiolabeled dCTP to label the newly synthesized fragments. In AP-PCR DNA fragments are size separated using PAGE and visualized via radiography (Welsh and McClelland 1990). It provides less number of bands detected as compared to RAPD and DAF.

2.7.1.3 DNA amplification fingerprinting (DAF)

In DNA Amplification Fingerprinting (Caetano-Anolles et al., 1991), even shorter primers (5-7bp) at higher concentrations (3-30mM) with either low or high stringency annealing temperature are used, more number of fragments are detected. PAGE is used to resolve the amplified products with silver staining technique. It is an ideal system for individual identification and fingerprinting. Because of more DNA fragments, the likelihood of observing polymorphism between strains is increased.
2.7.1.4 Inter simple sequence repeats (ISSR)

This technique is not sequence arbitrary *per se*, but requires only limited sequence information and developed using micro-satellites themselves as primers. This involves the use of single primer composed of di-, tri-, tetra- or penta-nucleotide. It involves amplification of regions between adjacent, inversely oriented microsatellites using a single SSR containing primer (Zietkiewicz *et al.*, 1994). Polymorphisms are found abundant among species. These are dominant markers though occasionally a few of them exhibit codominance. Using this strategy, Gupta *et al.* (1994) found that tetra-nucleotide repeat primers provided informative and moderately complex patterns, tri-nucleotidiseless informative whereas, di-nucleotide primers resulted in smeared products. Only one primer is used in a PCR at a time. The primers can be either unanchored or anchored at 5’ or 3’ or at both the ends with 1-4 bases extended in the flanking sequences. When two such sequences are present within an amplifiable distance and in inverted orientation, the intervening DNA segment within these two repeats will be amplified. As the ISSR technique amplifies a large number of DNA fragments per reaction, representing multiple loci across the genome, it is an ideal method for fingerprinting rice varieties and a useful alternative to single locus or hybridization-based methods (Goodwin *et al.*, 1997). Amplification in the presence of nonanchored primers also has been called microsatellite-primed PCR, or MP-PCR, (Meyer *et al.*, 1993). Such amplification does not require genome sequence information and leads to multilocus and highly polymorphous patterns (Zietkiewicz *et al.*, 1994; Tsumara *et al.*, 1996; Nagaoka *et al.*, 1997). Each band corresponds to a DNA sequence delimited by two-inverted microsatellite. LikeRAPDs,
ISSRs markers are quick and easy to handle, but they seem to have the reproducibility of SSR markers because of the longer length of their primers.

The great robustness of ISSR in repeatability experiment and their being less prone to changing band patterns with changes in constituent or DNA template concentration makes them superior to other readily available marker systems in investigation of genetic variability variation among very closely related individuals and in crop cultivars classification (Fang and Roose, 1997; Nagaoka and Ogihara, 1997) and variability studies e.g. genetic diversity in *berchemia berchemiaefolia*, a Rhamnaceae plant (Lee et al. 2003) and *ber* (Singh et al., 2007)

2.7.1.5 Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP), is universally applicable and highly reproducible method, which reveals very high levels of polymorphism (Vos et al., 1995). This technique is based on Selective Restriction Fragment Amplification of DNA fragments (SRFA), involving three steps (i) cutting genomic DNA with restriction enzyme(s), (ii) ligating double stranded adapters to restriction fragments, and (iii) amplifying selective restriction fragments using universal primers. During selective amplification, one of the paired primers is end labeled with $^{32}$p. The amplified products are normally separated on a denaturing polyacrylamide gel and visualized by exposure to X-ray film, but the technique has been adapted to fluorescent labeled primers and also automated. SRFA can be performed with a single restriction enzyme, but the results are achieved when two different enzymes, a rare cutter (e.g. Mse I) and a
frequent cutter (e.g. Eco RI) are used. AFLP is a powerful, reliable, stable and rapid assay with potential application in genome mapping, DNA fingerprinting and marker assisted breeding. This PCR-based technique permits inspection of polymorphism at a large number of loci within a very short period of time and requires very small amounts of DNA. The reproducibility of AFLPs ensured by using restriction site-specific adapters and adapter-specific primers with variable number of selective nucleotides under stringent amplification conditions. Due to their various advantages, AFLPs are considered as one of the best molecular markers of choice for genome mapping. However, they require more DNA, dominant in nature, complex banding pattern, use of radioactivity and anchoring the randomly distributed amplified bands to particular chromosomal region and are quite difficult compared to STMS (described later). Probably the single greatest advantage of the AFLP technology is its sensitivity to polymorphism detection at the total-genome level. With all of these assets, AFLP markers are molecular standard for investigations ranging from systematics to population genetics. This technique has been used in genetic diversity studies in *Musa spp.* (Ude et al., 2003), pear (Kim et al., 2005), *Cucurbita pepo* (Ferriol et al., 2003), Abyssinian banana (Negash et al., 2002) ber (Singh et al., 2006) and many other plant species.

2.8 Other Sequence Arbitrary Methods

Several variations of the above described methods are employed. Either by using more than one arbitrary primer (Callahan et al., 1993) or by using a degenerate primer in the amplification reaction (Caetano-Anolles, 1994), increasingly complex RAPD and
DAF amplification patterns has been observed. One approach to find increased polymorphism is the pre-digestion of the template DNA with restriction endonucleases termed as template endonucleases cleavage multiple arbitrary amplicon profiling (tecMAAP). Despite the fact that endonucleases digestion destruct potential amplicons, new amplicons containing higher levels of primer-template mismatch are amplified. Another approach to enhance the level of informativeness from DAF reactions is by using primers which contain both a 5’ mini-hairpin sequence and a short 3’arbitrary sequence either alone (Caetano-Anolles and Gresshoff, 1994) or in a two step amplification procedure called arbitrary signature from amplification profiles (ASAP) (Caetano-Anolles and Gresshoff, 1996). Using ASAP, a DAF amplification is first performed and then an aliquot of the resulting amplification reaction is used as template in a second amplification primed with either a mini-hairpin based primer or alternatively 5- anchored simple sequence repeat (SSR) primer (Caetano-Anolles and Gresshoff, 1996).

Two other methods have been described that combine aspects of RAPDs and SSRs. The first of these methods termed as random amplified microsatellite polymorphisms (RAMPs) (Wu et al., 1994), is an extension of anchored SSR method described by Zietkiewicz et al., (1994). By combining a radiolabeled, anchored SSR primer with a 10mer RAPD primer, informative fingerprinting patterns are observed. Because of the labeled anchored repeat primer, only those amplification products that contain the target repeat motifs are visualized. The other method involved hybridizing the SSR repeat primers to RAPD amplification patterns, is called either random
amplified hybridization microsatellites (RAHM) (Cifarelli et al., 1995) or random amplified microsatellite polymorphisms (RAMPO) (Richardson et al., 1995). In the technique, the bands observed following hybridization with the SSR probe were amplification products not observed following hybridization with the SSR probe were amplification product is not observed using only ethidium bromide staining (Richardson et al., 1995) and thus may uncover additional polymorphism.

2.9 Sequence-Dependent Markers

These techniques are based on PCR using two sequence-dependent oligonucleotide primers. Sequence information is necessary in order to design primers, which would facilitate successful amplification of a specific DNA fragment. Despite their considerable development cost and sequence-dependent, these PCR-based markers are now being exploited in many plant species. Allelic and codominant nature, highly specific, reproducibility and assay robustness make these markers especially attractive for marker-assisted plant breeding. This includes Sequence Tagged Sites (STS), Sequence Characterized Amplified Region (SCAR), and Simple Sequence Repeats (SSR) otherwise referred as micro-satellites

2.9.1 Sequence tagged sites (STS)

A sequence tagged site (STS), is a short stretch of genomic sequence that can be detected by PCR and mapped to a specific site as a landmark in the genome. They are essentially derived from RFLP probe sequences by designing 20-25bp primers using these sequences (Oslon et al. 1989; Inoue et al., 1994, Thomas and Scott 1993, Ribaut et
al., 1997). They are co-dominant in nature and generally having lower level of polymorphism compared to microsatellites. A crucial event in the history of the Human Genome Project was the decision to use sequence-tagged sites (STSs) as common landmarks for genomic mapping. Following several years of constructing STS-based maps of ever-increasing detail, the emphasis has recently shifted towards large-scale genomic sequencing (Schuler, 1998). A computational procedure called ‘electronic PCR’ allows STS landmarks to be revealed as data emerge from the sequencing pipeline, thereby bridging the gap between mapping and sequencing activities. Electronic PCR (e-PCR) refers to the process of recovering these unique sites in DNA sequences by searching for subsequences that closely match the PCR primers and have the correct order, orientation, and spacing that they could plausibly prime the amplification of a PCR product of the correct molecular weight (Schuler, 1997). Hudson et al. (1995) constructed physical map of human genome using 15,086 sequence tagged sites (STSS) with an average spacing of 199 kilobases. In the project, assembly of a radiation hybrid map of the human genome containing 5,264 loci and incorporated 5264 loci. The map provides radiation hybrid coverage of 99 per cent and physical coverage of 94 per cent of the human genome.

2.9.2 Expressed sequence tags (Est’s)

Expressed sequence tags (Est’s) are derived mainly from cDNA sequences isolated from particular tissue or developmental stage or under biotic or abiotic stress. They are functional sequences; Primers are designed based on these sequences. This is co-dominant marker and it played a great role in construction of physical maps for
whole genome sequences. The increasing availability of ESTs in the rice genome project (Sasaki, 1998), wheat (Leigh et al., 2003 and Nicot et al., 2004) and grape (Scott et al., 2000). They are abundant and distributed throughout the genome and could be a significant tool in gene mapping, tagging and cloning.

2.9.3 Sequence characterized amplified region (SCAR)

This is one modification of RAPD, which improves its reproducibility, SCAR (Sequence Characterized Amplified Region), in which the polymorphic band detected with RAPD are eluted, cloned and sequenced (Paran and Michelmore, 1993). Based on the sequence information, specific primer is then designed, which gives a highly reproducible banding pattern. The ability to observe polymorphism was dependent upon the SCAR. Some SCARs show dominant whereas, some other can show codominance (Paran and Michelmore, 1993). SCAR has successfully been used for tagging of male fertility restorer gene in Brassica juncea (Ashutosh, 2007) and gene for resistance to Plasmodium brasilense in Chinese cabbage (Piao et al., 2004)

2.9.4 Cleaved amplified polymorphic sequence (CAPS)

Instances, where direct gel electrophoresis of PCR products does not reveal polymorphism, may necessitate the use of more elaborate detection methods. CAPS are PCR amplification products which are subsequently cleaved using restriction endonucleases and size separated in order to reveal polymorphism (Konieczny and Ausubel, 1993). Due to the lower level of polymorphism detected using STS; cleaved amplified Polymorphic sequence (CAPS) is used as a STS modification for increasing
the level of polymorphism. The amplified product is digested with one or more suitable
restriction enzyme(s) to detect polymorphism in the enzyme recognition site. CAPS has
been done to unveil the polymorphism in *Brassica juncea* (Varshney *et al.*, 2004) and
for tagging the gene for resistance to *Plasmodium brassicae* in Chinese cabbage
(Piao *et al.*, 2004)

2.9.5 Single nucleotide polymorphism (SNP)

The single nucleotide polymorphism (SNP) refers to a specific and defined
position at a chromosomal site at which the DNA sequences of two individuals differ by
a single base. SNPs is the most abundant variation in the genome (Nasu *et al.*, 2002),
they can contribute directly to a phenotype or can be associated with a phenotype as a
result of linkage disequilibrium (Risch and Merikangas, 1996). SNPs are abundant, co-
dominant in nature and highly reproducible systems. They may be found in both-
transcribed and non-transcribed regions and in some instances are the direct cause for
observed phenotypic variation. In humans, SNPs have been found at a frequency greater
than 1 per 1000 base pairs. It has been used extensively to map, tag and cloning genes
of interest (Monna *et al.* 2002). Nasu *et al.* 2002 has developed 213 SNP markers
covering the entire rice genome using DNA sequences of three *japonica* cultivars, two
*indica* cultivars and one wild rice *O.rufipogon*. The success of marker assisted selection
(MAS) in the hands of plant breeders depends on the development of molecular marker
systems that are not only more efficient and cost effective, but are also amenable to
automation and high throughput approaches to handle large segregating populations.
SNPs have proven ideal for this purpose (Gupta *et al.*, 2001).
SNPs may be found in the non-repetitive coding or regulatory sequences as well as the repetitive non-coding ones. It is important here to focus on SNP discovery in or nearby coding sequences, this will make it more likely that the SNPs will either be located within the gene of interest or will be tightly linked with it. Several approaches are employed for SNPs discovery including alignment of genomic and Est sequences available from more than one source. This is highly cost effective way. It is also very attractive for developing trait specific SNPs if the location and the sequence is available. A whole genome shotgun sequence is another approach for the discovery. In this approach, random clones from a mixture of DNA from several genotypes are sequenced for several folds after which the sequences are aligned. In case of organisms that have bees used for genome sequencing such as rice, one of the common approaches is the alignment of the overlapping BACs and PACs and detect the mismatched SNPs. Reduced representation shotgun (RRS) approach is yet another way for SNP discovery. In this approach, equal amounts of DNA from different genotypes are mixed, completely digested, electrophoresed and 500-600bp bands are excised, cloned and sequenced, alignment of the sequences from same loci will reveal the possible candidate SNPs for this particular loci. The second part of SNPs is the genotyping, which can be classified, in two broad approaches, gel-based and non gel-based SNP detection. The first part includes the use of RFLP or AFLP in case the SNP is altering restriction endonucleases recognition site, the use of Single Strand Confirmation Polymorphism (SSCP), allele specific amplification using allele specific primers is done. The non gel-base approach is the fast growing approach including Taqman assay, molecular
beacons, oligonucleotide ligation assay, DNA chips and microarrays, dynamic allele-specific hybridization, minisequencing and genetic bit analysis, temperature modulated heteroduplex analysis, masscode™ system, MegaBACE SnuPe Genotyping Kit, Amersham Pharmacia Biotech, Inc, USA. And invasive cleavage assay

2.9.6 Simple sequence repeats (SSRs)

Microsatellite DNA sequences were first studied in humans, where they were found to be abundant and dispersed throughout the genome (Hamada and Kakunga 1982; Hamada et al. 1982). Since that time, they have been found in a wide array of other eukaryotes including several monocot and dicot plant species including rice. Microsatellites consist of tandemly arrayed di-, tri- and tetra-nucleotide repeats, and are hypervariable and ubiquitously distributed throughout eukaryotic genomes. Among different crops species, the frequencies and occurrence of the most common dinucleotide repeats [(AC)ₙ and (GA)ₙ ] have been worked out in relatively greater detail. (AAG)ₙ and (AAT)ₙ are the most frequent trinucleotide repeats in plants (Gupta et al., 1996). Human genome is estimated to contain an average 10-fold more microsatellites than plant genome (Powell et al., 1996). Microsatellites are not clustered in specific regions but rather uniformly distributed in different regions. Tomato is an exception in this regard as it shows clustering of microsatellite (Gupta and Varshney, 2000). Primers are designed using unique sequences on both sides of the flanking regions of the repeat motif region to avoid the complex multiple pattern, since these repeats are present up to thousand times throughout the genome, this ensures STMS to provide a simple banding pattern with a high level of polymorphism detected (Litt and
Lutty, 1989; Tautz, 1989; Weber and May, 1989) The high level of polymorphism detected is basically due to the difference of number of repeats in each individual. In several crop plants, including soybean (Akkaya et al., 1992), rice (Wu and Tanksley, 1993; Akagi et al., 1997), barley (Becker and Heun, 1995), wheat (Roder et al., 1995), maize (Senior and Heum, 1993), brassica (Langercrantz, 1993) and fruit species like grapes (Scott et al., 2000), citrus, specific amplification of microsatellite loci has indicated that microsatellite DNA markers are more variable than RFLP markers. This new type of molecular markers, known also as simple sequence repeats (SSR) or simple sequence length polymorphism (SSLP) has been developed based on DNA sequence variation, is based on a 1-6 nucleotide core element that is tandemly repeated from two to many thousands of times. (Hamada et al. 1982, Litt et al. 1989; Tautz et al. 1986; Stallings et al. 1991; Akkaya et al., 1992) A different “allele” occurs at an SSR locus as a result of changes in the number of times a core element is repeated, altering the repeat region. They are co-dominant in nature, so very ideal for segregating populations. Differences in length at an STMS locus are detected with DNA amplification by the PCR using two oligonucleotide primers that complement unique sequences flanking the SSR locus. Sizes of the amplified products are then precisely determined by electrophoresis in either agarose or Polyacrylamide gels with detection by EtBr staining, autoradiography (using a single $^{32}$p- labeled primer) or fluorescence (using a fluorescent labeled primer). Current research has suggested that the length variations between alleles at an STMS locus are created by slippage of DNA polymerase during the
replication of the tandem repeat followed by a failure of DNA mismatch repair enzymes to restore the original sequence (Strand et al., 1993)

STMS can provide more information more easily than previous DNA-based genetic marker technologies, such as restriction fragment length polymorphism (RFLP) and randomly amplified DNA (RAPD). It is also sensitive, only a small quantity of DNA is required, analytically simple, data are unambiguously scored, and highly reproducible, broadly applicable, loci are frequently conserved between related species and sometimes across genera (Moore et al., 1991), readily transferable, information can be communicated as simple sequences of primer pairs, and does not require the physical transfer of probes among laboratories; flexible, these markers can be used as sequence tagged sites to provide anchors between genetic linkage maps and physical chromosome locations. The only significant limitation of SSR analysis may be the initial investment and the technical expertise required to clone and sequence the loci, which is not the case in rice since the whole genome sequence is now available. In silico identification of STMS loci becomes quite easy. Once primer sequences are designed and published, analysis of SSR loci will be practical for any laboratory capable of PCR and electrophoresis. The throughput and cost effectiveness of screening loci also could be greatly improved by multiplex PCR, which allows the simultaneous amplification and scoring of multiple STMS loci in a DNA sample in a single PCR reaction and a single lane of gel electrophoresis.

Through surveys of sequences in EMBL and Gene Bank databases, it was discovered that all possible di- and trinucleotide repeat motifs were present at 5- to 10-
fold greater frequencies than expected by a random distribution. The only exception being (CG)n which occurred at significantly less than a random frequency. Other forms of imperfect and scrambled arrangements of repeat units ("cryptic repeat") also were extremely common in eukaryotic genomes. Slippage during DNA replication was proposed as a mechanism for the creation and hypervariability of these repeat elements. Regions of divergence between conserved genes in closely related species often contained these simple repeats. It was hypothesized by Tautz (1989) that because STMS were so prevalent in the genome and so highly variable, mutations at these sites may be a more important source of evolutionary variation than classical point mutations and chromosomal translocations. The information content of STMS loci as a genetic marker is directly proportional to the number and frequency of alleles present in a population. It is known as the percentage of polymorphism or polymorphism information content (PIC) value. In a study of 100 (AC)n loci, Weber and May (1989), reported that 64% were perfect tandem repeats, 25% were imperfect, and 11% compound. The information content of the loci was directly correlated with the number of tandemly repeated units. PIC values ranged from close to 0 at n=10 up to 0.8 at n=24. As such, it was estimated that the human genome potentially contains ~7000 (AC)n loci with PIC >0.7.

2.10 Development of Microsatellite Markers

There are a number of ways to obtain microsatellite markers. Screening genomic libraries by hybridizing with SSR probes and sequencing the hybridized positive clones is traditional but a laborious and costly approach. While screening the SSR-enriched, small-
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insert libraries can significantly reduce time and cost (Billotte et al., 2001). Enrichment can be realized by hybridizing SSR-containing fragments with biotin-labeled probes that are either captured by magnetic beads coated with streptavidin or fixed on a nitrate filter (Edwards et al., 1996). The eluted portion after removing non-hybridized DNA would be highly enriched for microsatellites, with enrichment efficiency between 50–90% (Butcher et al. 2000).

Ostrander et al. (1992) described an efficient method for the construction of small-insert genomic libraries enriched for highly polymorphic, simple sequence repeats. With this approach, libraries in which 40-50% of the members contain (CA)$_n$ repeats are produced, representing an ≈ 50 fold enrichment over conventional small insert genomic libraries. Briefly a genomic library with an average insert size of less than 500 base pairs was constructed in a phagemid vector. Amplification of this library in a dut ung strain of Escherichia coli allowed the recovery of the library as closed circular single-stranded DNA with uracil frequently incorporated in place of thymine. This DNA was used as template for second-strand DNA synthesis, primed with (CA)$_n$ or (TC)$_n$ oligonucleotides, at elevated temperatures by a thermostable DNA polymerase. Transformation of this mixture into wild-type E. coli strains resulted in the recovery of primer-extended products as a consequence of the strong genetselection against single-stranded uracil containing DN molecules and recovered a library that was enriched for targeted microsatellite-containing sequences. They suggested the use of this method to generate marker-selected libraries nearing any simple sequence repeat from cDNAa,
whole genomes, single chromosomes or more restricted chromosomal regions of interest.

Kandpal et al. (1994) described a simple and rapid method for constructing small-insert genomic libraries highly enriched for dimeric, trimeric and tetrameric nucleotide repeat motifs. This approach involves use of DNA inserts recovered by PCR amplification of a small-insert sonicated genomic phage library or a single-primer PCR amplification of Mbo I-digested and adaptor-ligated genomic DNA. The genomic DNA inserts are heat denatured and hybridized to a biotinylated oligonucleotide. The biotinylated hybrids are retained on a vectrex-avidin matrix and eluted specifically. The elute is PCR amplified and cloned. More than 90% of the clones in a library enriched for (CA)$_n$ microsatellites with this approach contained clones with inserts containing CA repeats. They also used this protocol for enrichment of (CAG)$_n$ and (AGAT)$_n$ sequence repeats and for Not I jumping clones.

Li et al. (2001) constructed microsatellite-enriched libraries to isolate microsatellite sequences in Avena species and oat cultivars. One hundred clones were isolated and sequenced from three oat microsatellite-libraries enriched for either (AC/TG)$_n$, (AG/TC)$_n$ or (AAG/TTC)$_n$ repeats of these, seventy eight clones contained microsatellites. A database search showed that 42% of the microsatellite flanking sequences shared significant homology with various repetitive elements. Alu and retrotransposon sequences were the two largest groups associated with the microsatellites. Forty four primer sets were used to amplify the DNA from 12 Avena species and 20 Avena sativa cultivars. Sixty two percent of the primers revealed
polymorphism among the *Avena* species, but only 36% among the cultivars. In the cultivars, the microsatellites associated with repetitive elements were less polymorphic than those not associated with repetitive elements. Only 25% of the microsatellites associated with repetitive elements were polymorphic, while 46% of the microsatellites not associated with repetitive elements showed polymorphism in the cultivars.

Kolliker *et al.* (2001) developed simple sequence repeat (SSR) markers for white clover (*Trifolium repens* L.). They sequenced 1123 clones from genomic libraries enriched for (CA)$_n$ repeat yielded 793 clones containing SSR loci. The majority of SSRs consisted of perfect dinucleotide repeats, only 7% being trinucleotide repeats. After exclusion of redundant sequences and SSR loci with less than 25 bp of flanking sequence, 397 potentially useful SSRs remained.

Aranzana *et al.* (2002) developed a genomic libaray enriched with AG/CT repeats from the peach cultivar ‘Merrill O’Henry’ and observed that 61% of the clones carrying a microsatellite sequences and a yield of one polymorphic microsatellite every 2.17 sequenced clones. From 35 microsatellite detected, 24 were polymorphic in a set of 25 cultivars. A total of 82 alleles were found with the polymorphic microsatellites with an average of 37% of observed heterozygosity.

He *et al.* (2003a) developed microsatellite markers in cultivated peanut using the SSR enrichment procedure and observed that the GA/CT repeat was the most frequently dispersed microsatellite in peanut. The primer pairs were designed for fifty-six different microsatellites, 19 of which showed a polymorphism among the genotypes studied.
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Ahmad et al. (2003) evaluated the potential of microsatellite markers for use in Citrus genome analysis. Microsatellite loci were identified by screening enriched and nonenriched libraries developed from ‘Washington Navel’ Citrus. Microsatellite-containing clones were sequenced and 26 specific PCR primers were selected for cross-species amplification and identification of cultivars/clones in Citrus. After an enrichment procedure, on average 69.9% of clones contained dinucleotide repeats (CA)n and (CT)n, in contrast to <25% of the clones that were identified as positive in hybridization screening of a nonenriched library. A library enriched for trinucleotide (CTT)n contained <15% of the clones with (CTT)n repeats. Repeat length for most of the dinucleotide microsatellites was in the range of 10 to 30 units. We observed that enrichment procedure pulled out more of the (CA)n repeats than (CT)n repeats from the Citrus genome. All microsatellites were polymorphic except one. No correlation was observed between the number of alleles and the number of microsatellite repeats.

Ritschel et al. (2004) developed microsatellite markers using enriched genomic library in melon (Cucumis melo L.) and cucurbit species. They identified seven hundred clones containing microsatellite sequences from a Tsp-AG/TC microsatellite enriched library, from which they designed one-hundred and forty-four primer pairs. When 67 microsatellite markers were tested on a panel of melon and other cucurbit accessions, 65 revealed DNA polymorphisms among the melon accessions. For some cucurbit species, such as Cucumis sativus, up to 50% of the melon microsatellite markers could be readily used for DNA polymorphism assessment, representing a significant reduction of marker development costs.
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Giraldo et al. (2005) developed 26 polymorphic microsatellites from a genomic library of fig (Ficus carica L., Moraceae, cv. Cuello de Dama Blanco) enriched for CT/AG repeats and found that enrichment procedure was highly successful (>60% of the clones sequenced contained microsatellite sequences). Microsatellite polymorphism was evaluated in 15 fig cultivars from different geographical areas. The mean expected and observed heterozygosities over the 25 single-locus microsatellites averaged 0.42 (range of 0.12-0.77) and 0.47 (range of 0.13-0.93) respectively. The total value for the probability of identity was $2.81 \times 10^{-8}$. The low values of variability and the absence of clear groups in the genotypes studied and indicated a narrow genetic base in cultivated common fig. The developed microsatellite primers were also observed for high level of cross-species transferability in Morus species.

2.11 Use of Microsatellite Markers

The application of STMS marker analysis to plant genetics is only just beginning, but is being adopted very rapidly throughout the research community. A number of loci have been characterized in agricultural species including rice, soybean, maize, barley, rapeseed, and grape. In each case, preliminary work involved identifying STMS loci in existing sequence database, creating primer pairs for these loci, and surveying a small set of diverse germplasm for polymorphism. Subsequent efforts have involved cloning and sequencing STMS loci by screening size-fractionated genomic libraries, and using primer pairs flanking STMS loci to survey sets of individuals representing both agronomically important and distantly related species.
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The analysis of STMS alleles in plant DNA is being greatly advanced by the capabilities for automated sizing of PCR products by GeneScan™ fluorescence-based detection (Applied Biosystems Division of Perkin-Elmer). Differences in allele sizes of only two nucleotides can be resolved reproducibly. A multiplex of different primer pairs tagged with fluorescent dyes of different colors allows multiple loci to be analyzed in a single PCR reaction and a single gel lane. This technology ultimately may be complemented with automated DNA extraction and PCR set-up, permitting very high rates of sample throughput and low unit cost for large scale operations, such as genetic resources profiling or scoring segregation in marker assisted breeding programs. STMSs will be useful in the study of genetic organization and variation in a myriad of ways. Their ease of use and high information content naturally will lead to the complementation and replacement of other types of genetic markers in many situations, but possibly also to novel applications not previously considered for DNA-based markers (Brown et al., 1996). The application of STMS markers to varietal identification and for plant breeders and seed producers' right protection is already underway (Smith, 1994).

Identification of markers linked to useful traits has been based on construction of complete linkage maps and the study of co-segregation, or bulk segregation analysis (BSA) in case of simple traits. However, alternative methods such as the construction of partial maps and combination of pedigree and marker information have also proved useful in identifying marker/trait association. Molecular markers have been looked upon as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. They have also become
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extremely popular markers for phylogenetic analysis adding new dimensions to the evolutionary theories. If we look at the history of the development of these markers, it is evident that they have been improved over the last two decades to provide easy, fast and automated assistance to scientists and breeders. Genome analysis based on molecular markers has generated a vast amount of information and a number of databases are being generated to preserve and popularize it. Microsatellite markers, especially STMS markers, have been found to be extremely useful in this regard. Owing to their quality of following clear Mendelian inheritance, they can be easily used in the construction of linkage maps, which can provide an anchor or reference point for specific regions of the genome.

Allele mining focuses on the detection of allelic variation in important genes and/or traits within a germplasm collection (Simko et al., 2004). If the targeted DNA (either a gene of known function or a given sequence) is known, then the allelic variation (usually point mutations) in a collection can be identified, using methods developed for the purpose. (Lemieux et al., 1998). Association studies of artificial progenies are an alternative to segregation analysis for identifying useful genes by correlation of molecular markers and a specific phenotype (Gebhardt et al., 2004). Association studies can be performed on a germplasm collection and also on other materials, as long as significant linkage disequilibrium (LD) exists, for example, breeding materials. It may be especially useful for those crops where appropriate populations for genetic analysis cannot be obtained or their production is too time-consuming (Simko et al., 2004). It is also useful for those crops for which sequence information does not exist and is unlikely
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to be available soon. Knowledge of molecular information in major crops and species and of the synten of genomes, especially conservation of gene order, has also opened up prospects for identifying important genes or variants in other crop types, particularly those that receive little attention from formal research.

2.12 Genetic Diversity Studies in Ber

Genetic variability is a pre-requisite for genetic improvement in any breeding programme. Since reports on genetic diversity studies on Ziziphus using molecular mark es are very scanty (Devanshi et al., 2007), in the review the fruit and woody trees in general will be detailed for the molecular studies. Molecular marker technology has wide and diverse applications they can broadly be classified into two main categories. The first category is genome analysis applications, which include mapping, tagging, map based cloning, gene pyramiding and marker-assisted selection (MAS) for genes of interest in both simple as well as quantitative traits (Wu and Tanksley, 1993). The second category is the fingerprinting applications including varietal identification, ensuring seed purity, phylogeny and evolution studies, diversity analysis, elimination of germplasm duplicates. The DNA markers are more widely employed for molecular tagging of genes and molecular marker assisted selection (Gupta et al., 1999). Microsatellite markers have been used to distinguish the inter-varietal chromosome substitution lines of wheat (Korzun et al., 1997).

Prevost and Wilkinson (1999) tested two statistical functions, marker index and resolving power for their correlation to the proportion of genotypes identified but is
independent of the number of the genotypes studied in their study on fingerprinting of potato cultivars using ISSR markers. Marker index failed to correlate significantly with genotype diagnosis but a strong and seemingly linear relationship was observed between resolving power of a primer and its ability to distinguish genotypes ($r^2=0.98$). Resolving power of one or a pair of primer was found to provide a moderately accurate estimate of the number of genotypes identified. The marker index did not show any significant relationship between MI and the number of genotypes identified whereas, a linear relationship was observed between Rp and the proportion of cultivars identified.

Oh et al. (1988) suggested that Korean jujube (Z. sativa) strains could be classified with allozyme analysis using glutamate oxalacetate transaminase (GOT), esterase and peroxidase systems. Further analysis, however, showed that use of allozymes did not always show satisfactory results.

Vashishtha et al. (1989b) analysed the flavonoid distribution patterns of 59 ber cultivars along with the wild relatives, jhar (Z. nummularia) and boradi (Z. mauritiana var. rotundifolia). While no flavonoids in the wild relatives were observed to be common with those in the Z. mauritiana cultivars, similarities were recognised among the ber cultivars. The flavonoid patterns of many of the cultivars did not agree with the observed relationships based on their morphological characteristics, e.g. cultivars Ajmeri, Umran and Katha had identical flavonoid patterns and morphological characters, whereas round fruited cultivars such as Gola, Gola Gurgaon, Popular Gola and Kakrola Gola were dissimilar in their flavonoid patterns. Based on the distinctly different flavonoid patterns,
cultivars Illaichi, Willaiti, Mirchia and Sanaur-3 were recognised as chemical races (Vashishta et al., 1989a).

Weekly et al. (2002) studied genetic diversity in Z. celeta using random amplified polymorphic DNAs (RAPDs) to investigate genetic variability. One hundred and ninety-nine unique stem samples collected from one *ex situ* and five *in situ* populations were assayed for the presence or absence of a band for 32 RAPD markers. Based on unweighted pair-group mean cluster analysis (UPGMA), only 11 multi-locus genotypes (MLGs) were identified. Eight of these MLGs correspond to MLGs identified in an earlier allozyme study.

Meghala et al. (2005) studied genetic diversity among Indian sapota (*Manilkara zapota*) cultivars using RAPD markers and reported high genetic variability in Indian sapota cultivars might have originated through seedling segregation, intercrossing among cultivars or because a large number of cultivars/genotypes were introduced. It is also possible that both of these factors might have contributed to the genetic diversity in this crop.

Koller et al. (1993) differentiated 11 apple cultivars using random amplified polymorphic DNA (RAPD) markers and suggested that it would be possible to establish a standard set of primers that can be used to distinguish and characterize most of the common apple cultivars.

Schnell (1993) analysed 9 *Mangifera species* to determine phylogenetic relationship using 10 RAPD markers. One hundred nine bands were resolved and the
cluster analysis suggested that within Mangifera section, M. casturi, M. quartifida and M. torquenda are closely related, but M. indica and M. laurina are quite distant from the others. Within the subgenus Limus, M. foetida and M. pajang are genetically close; M. decandra and M. odorata are more distant.

Stiles et al. (1993) analysed relationships among ten cultivars of papaya (Carica papaya L.) using RAPD markers. The 11 RAPD primers amplified a total of 102 distinct fragments. The genetic similarity coefficient ranged from 0.7 to 0.95 that suggested a narrow genetic base for domesticated papaya.

Jarret et al. (1997) analysed simple sequence repeat markers in 33 watermelon (Citrullus lanatus (Thunb.) Matsum. & Nakai) accessions to study genetic relationship. They reported substantial increase in the ability to differentiate among these accessions by using SSR markers as compared to enzyme polymorphism (Biles et al., 1989) but similar to RAPD polymorphism (Lee et al., 1996).

Fang et al. (1997) worked on fingerprinting of trifoliate orange germplasm accessions with isozyme, RFLP and inter-simple sequence repeat markers and reported that ISSR markers offer great potential for differentiating closely related citrus cultivars. They suggested that it may be possible to differentiate most mutationally derived citrus cultivars by using ISSR markers as probes in RFLP analysis.

Fang et al. (1998) used ISSR markers to study phylogenetic relationships among 46 Citrus L. accessions representing 35 species. The 10 primers generated 642 polymorphic fragments and grouped the 46 accessions into five clusters. Relationships
among the studied Citrus accessions revealed by the ISSR markers were in agreement to the previous taxonomic classifications.

Huang and Sun (2000) studied genetic diversity and relationships of 40 accessions of Ipomoea, representing 10 species of series of Batatas using ISSR markers. On average, 52 bands per accession were generated with most of the primers containing dinucleotide repeats. The ISSR analysis showed 62.2% polymorphism among the 40 accession studied. Of the species, examined I. trifida was found to be the most closely related to cultivated sweetpotato, the hexaploid I. batatas, while I. ramosissima and I. umbraticola were the most distantly related to I.batatas within the series.

Luro et al. (2000) studied genetic diversity among Citrus species using simple sequence repeats (SSRs). In the study, the genetic relationships among 73 individuals of 8 more economically important species of the Citrus genus (C. reticulata Blanco, C. sinensis (L.) Osb., C. aurantium L., C. paradisi Macf., C. maxima (Burm.) Merr., C. limon (L.) Burm., C. aurantifolia (Christm.) Swing. and C. medica L.) were estimated by the amplification of 10 loci of SSR. Polymorphism of 8 STMS was sufficient so as to obtain genetic aggregations where three main groups were clearly identified: the orange-mandarin group, the pummelo-grapefruit group and the lime-citron-lemon group. These Citrus species relationships were in large agreement with those established by previous analyses based on molecular markers and morphological traits, suggesting that SSR data will be useful for exploring infra-generic Citrus genetic taxonomy.
Degani et al. (2001) studied genetic relationship in strawberry using AFLP and RAPD markers and observed that RAPD markers successfully distinguished between 41 strawberry cultivars. They reported that RAPD-derived genetic similarity values to be better correlated with the coefficients of ancestry than the AFLP-derived genetic similarity values and suggested selective use of certain AFLP primer pairs which could be shown to give good genome coverage in strawberry might improve the utility of AFLP markers for predicting genetic relationships.

Kolliker et al. (2001) designed and characterize primers for 117 SSR loci and PCR products in the expected size range were amplified from 101 loci. These markers are highly polymorphic. 88% detecting polymorphism across seven white clover genotypes with an average allele number 4.8. Four primer pairs were tested in F2 population revealing Mendelian segregation. Successful cross-species amplification was achieved in at least one out of eight legume species for 46 of 54 primer pairs, although rate of successful amplification was significantly higher for Trifolium species when compared to species of other genera.

Li et al. (2001) evaluated level of polymorphism of microsatellite developed by them in Avena species and oat cultivars. An average of four alleles with polymorphism information content (PIC) of 0.57 per primer set was detected among the Avena species, and 3.8 alleles with a PIC of 0.55 among the cultivars. In addition, 54 barley microsatellite primers were tested in Avena species and 26% of the primers amplified microsatellites from oat.
Herrera et al. (2002) characterize genetic variation between *Vitis vinifera* cultivars using RAPD and ISSR markers. Both the marker system distinguished between the cultivar studied, although the resolving power of ISSR profiles was higher than that of RAPDs. They suggested use ISSR as an alternative cheap and technically simple approach that has high levels of repeatability.

Ahmad et al. (2003) observed 118 putative alleles generated through newly developed 26 SSR primer pairs using microsatellite enrichment library method in *Citrus*. The number of putative alleles per primer pair ranged from 1 to 9 with an average of 4.5. Microsatellite markers discriminated sweet oranges (*Citrus sinensis* (L.) osb), mandarin (*Citrus reticulata* Blanco), grapefruit (*Citrus paradisi* Macf.), lemon (*Citrus limon* (L.) Burm.f.), and citrane (hybrids of trifoliate orange and sweet orange), at the species level, but individual cultivars/clones within sweet oranges, mandarins and grapefruit known to have evolved by somatic mutation remained indistinguishable. They suggested that since these microsatellite markers were conserved within different *Citrus* species, they could be used for linkage mapping, evolutionary and taxonomic study in *Citrus*.

He et al. (2003a) in their study on peanut microsatellite primers observed the average number of amplified alleles per locus was 4.25, and up to 14 alleles were found at one locus. He et al (2003a) confirmed that microsatellite DNA markers produce a higher level of DNA polymorphism than other DNA markers in cultivated peanut.
He et al. (2003b) generated five hundred DNA sequences of tomato and searched for SSRs in these to design PCR primers. They screened 158 pairs of SSR primers screened against a set of 19 diverse tomato cultivars, 129 pairs produced the expected DNA fragments in their PCR products, and 65 of them were polymorphic with the polymorphism information content (PIC) ranging from 0.09 to 0.67. Among the polymorphic loci, 2-6 SSR alleles were detected for each locus with an average of 2.7 alleles per locus; 49.2% of these loci had two alleles and 33.8% had three alleles. The vast majority (93.8%) of the microsatellite loci contained di- or tri-nucleotide repeats and only 6.2% had tetra- and penta-nucleotide repeats. It was also found that TA/AT was the most frequent type of repeat, and the polymorphism information content (PIC) was positively correlated with the number of repeats. The set of 19 tomato cultivars were clustered based on the banding patterns generated by the 65 polymorphic SSR loci. Since the markers developed in this study are primarily from expressed sequences, they can be used not only for molecular mapping, cultivar identification and marker-assisted selection, but for identifying gene-trait relations in tomato.

Mengjun (2003) studied genetic diversity in chinese jujube (Ziziphus jujuba Mill.), and observed that it has high diversity in the levels such as chromosome karyotype (1A, 2A, 3A, 1B, 2B, 2C); shape, size (11.5-22.1μ×21.0-26.3μ) and surface sculpture of pollen; leaf length (2.3-10.1cm) and flower diameter (2.9-10mm); especially the shape, colour, weight (2-46g), growth period (60-145d), soluble solid (17-45%), Vc (61-1174mg/100g.fw) and cAMP content (2-302nmol/g.fw) of fruit; as well as the RAPD and
 isoenzyme (7 kinds) patterns. Diversity within some cultivars was also revealed by RAPD.

Ude et al. (2003) used 15 AFLP primer pairs and 60 RAPD primers to detect polymorphism and access genetic relationships in 25 plantains (Musa spp. subgroup AAB) from diverse parts of Western and Central Africa. They reported superiority of AFLP technique over RAPD technique based on greater PIC values for the primers of the former. A small group of cultivars of Cameroon were separated from the bulk of the plantains and based on that suggested to harbour useful genes from Cameroon for widening the genetic base of breeding population derived from the plantains.

Awasthi et al. (2004) studied genetic diversity and interrelationships among twelve domesticated and three wild species of the genus Morus (mulberry) using RAPD and ISSR markers. They observed that RAPD analysis using 19 random primers generated 128 discrete markers ranging from 500-3000 bp in size. One-hundred-nineteen of these were polymorphic (92%), with an average of 6.26 markers per primer. Among these were a few putative species-specific amplification products which could be useful for germplasm classification and introgression studies. The ISSR analysis employed six anchored primers, 4 of which generated 93 polymorphic markers with an average of 23.25 markers per primer. Cluster analysis of RAPD and ISSR data using the WINBOOT package to calculate the Dice coefficient resulted into two clusters, one comprising polyploid wild species and the other with domesticated (mostly diploid) species.
Ritschel et al. (2004) tested a random sample of 25 microsatellite markers to characterize 40 accessions of melon, generating an allelic frequency database for the species. The average expected heterozygosity was 0.52, varying from 0.45 to 0.70, indicating that a small set of selected markers should be sufficient to solve questions regarding genotype identity and variety protection. Genetic distances based on microsatellite polymorphism were congruent with data obtained from RAPD marker analysis.

Zhuang et al. (2004) studied genetic relatedness among Cucumis spp. using SSR and RAPD marker systems. They observed a total of 109 SSR and 398R RAPD bands and analysed these for genetic relationships through cluster analysis. From the study they reported that these two marker systems are highly concordant with correlation between SSR and RAPD genetic distance (GD) estimates \( r = 0.94 \). SSR and RAPD analysis of 22 accessions grouped them into two distinct groups designated as CS and CM. Within the CS group, 11 *C. sativus* genotypes, *C. hysticus* and *C. hystrix* accessions, whereas, in the group CM six *C. melo* genotypes and *C. metuliferus* were grouped.

Zhang et al. (2005) tested RAPD markers for relationship studies in tobacco (*Nicotiana tabacum* L.) cultivars and observed clear pattern of division among the flue-coloured tobacco accessions based on geographic origin. They reported that RAPD is an effective tool for flue-coloured tobacco germplasm management, cultivar protection and cultivar improvement.

Singh et al., (2006b) studied genetic diversity among ber (*Ziziphus* spp.) genotypes using AFLP markers. They reported that 11 primer pairs detected 952
Review of literature

fragments of which 789 (83.8%) were polymorphic. Similarity coefficients ranging from 0.14-0.86 suggested the divergence among Ziziphus accessions. Cluster analysis revealed complete separation of the accessions of the cultivated and the wild species into two distinct groups. Morphologically similar cultivars often remain clustered together with high degree of similarity. High correlation of the similarity matrix obtained with a single primer combination with that based on all the eleven primer combinations suggested utility of one or a few primer combinations in the estimation of diversity in ber. Nine of the primer combinations detected many accession specific amplified fragments and individually gave a discrimination rate of 1. Any one of these informative primers thus can be used for maintenance of the quality of both the scions and the rootstocks for establishing productive orchards.

Devanshi et al. (2007) studied genetic relationship among 50 ber genotypes representing Z. mauritiana, Z. nummularia and Z. spina-christi using RAPD markers. Out of 120 primers initially tested, 46 were highly reproducible and generated 368 RAPD markers with 86.2% polymorphism (316 polymorphic bands). The number of amplification product per primer ranged from 2 to 17 with an average of 8 bands per primer. The resolving power (Rp) for different primers ranged from 0.48 to 9.37 and polymorphic information content (PIC) ranged from 0.12 to 0.82. Nineteen primers distinguished at least one genotype that would prove to be highly useful for identification of genotype and designing future breeding strategy. Genetic relationships between the accessions were established based on Jacquard’s similarity coefficient and it ranged from 26.3% to 78.9% suggesting that the Ziziphus germplasm is genetically diverse. The
present study has proved that ber genotype earlier reported to be similar based on morphology are genetically different. They suggested that RAPD analysis is an efficient marker technology for delineating genetic relationships among genotypes and estimating genetic diversity, thereby enabling the formulation of appropriate strategy for conservation and improvement programmes.

Singh et al. (2007a) studied bitter gourd (*Momordica charantia* L.) for the genetic diversity based on inter-simple sequence repeat (ISSR) markers and reported the number of polymorphic markers ranged from 0 (UBC 841) to 12 (UBC 890) with a mean of 6.27 markers per primer. Pair-wise genetic distances (GD) of the 38 bitter gourd accessions, based on the 125 markers, ranged from 0.093 (‘Pusa Do Mausami’- green vs. ‘DBTG 7’) to 0.516 (‘Pusa Do Mausami’- white vs. ‘DBTG 101’) and suggested a wide genetic base for these genotypes.

Singh et al. (2007b) studied genetic diversity among 47 ber accessions belonging to cultivated species (*Ziziphus mauritiana* Lam) and one wild accession of *Ziziphus nummularia* (Burm f) using inter-simple sequence repeat (ISSR) markers. A total of 167 amplification products were detected with 18 ISSR primers of which 152 (89.96%) were polymorphic. Most of the primers that produced distinct bands (14 primers out of 18) contained dinucleotide repeats. Primers based on (AC)_n and (AG)_n repeats produced more polymorphic bands. Genetic similarity ranging from 43.07% to 90.30% suggested that the 48 *Ziziphus* genotypes used in the study were divergent. Cluster analysis based on UPGMA method and Bootstrap analysis separated all the 48 genotypes in four distinct clusters. They reported that morphologically similar genotypes can be distinguished and
Review of literature emphasized the use of molecular markers to the taxonomists. Morphologically similar but genetically distinct genotypes, identified using ISSR markers could be potential sources for genotype identification and to resolve controversies over misnomination of *ber* genotypes.