INTRODUCTION

*Piper* is the largest and most diverse genus in the family Piperaceae and comprises about 2000 species that are economically and ecologically important (Mabberley, 1997; Sing, 2000; Jaramillo and Manos, 2001, 2006; Parthasarathy et al., 2006). The diversification of this genus is of interest to agricultural science as it holds clues in understanding genetic linkages and evolution of peppers. *Piper* species have a pantropical distribution and are the most commonly found understory of tropical rainforest. The wide diversity of its characteristic features as well as the medicinal importance of the genus makes it an obvious model for studies on genetic diversity. India seems to be the Centre of origin for about 110 species of Piperaceae (Karthikeyan, 2000). The genus *Piper* in India has 50 species only two of which are monoecious and the remaining dioecious (Hooker, 1890; Santapau and Henry, 1994). Of these dioecious species 26 are endemic to the Himalayan region and 8 species are endemic to the peninsular region (Nayar, 1996). There are 16 species reported from Kerala and 19 species from Tamil Nadu (Henry et al., 1987; Sasidharan, 2004).

From time immemorial India has been recognized the world over as the home of spices. Once considered a luxury, spices today have become an integral part of our daily diet. One of the most valuable spices is the pepper (*Piper nigrum*) - once known as the black gold. Next to *Piper nigrum* the most important medicinal spice is *Piper longum* (Fig.1) (Indian long pepper) which is dioecious in nature and has commercial significance in the Indian subcontinent. The pungency of the fruits of long pepper is mainly due to the piperidine alkaloid piperine. Long pepper is the accepted source of drugs pippali and pippalimulam throughout India. While pippali is the dried ripe fruits, pippalimulam is the root (Sivarajan and Indira, 1994).

*Piper longum* has different names in every language of India. Pipli, Pipar, Pipal (Hindi), Hippali, Thippali balli (Kannada), Tippali, Pippali (Malayalam), Pimpli
(Marathi), Pipli, Tippili (Tamil), Pippallu, Pippali (Telugu), Pippali, Magadhi, Kana, Ushana, Pippali (Sanskrit) and Indian long pepper (English) are the different names (Oommen et al., 2000). The fruits are used as spice and pickle. The fruits have a pungent taste and cause salivation and numbness in the mouth. The pharmacological properties of different plant parts are well known in various traditional systems of medicine especially in ayurveda, and have been validated by modern scientific research (Neelam and Krishnaswamy, 2000).

**BOTANICAL DESCRIPTION AND DISTRIBUTION OF* PIPER LONGUM***

*P. longum* is an aromatic slender, sub-scandent herb or occasional climber with perennial woody roots, creeping under shrub, growing generally in the areas of hotter and humid climate (Fig. 2). The erect shrub has a thick, jointed and branched rootstock. The stems are jointed. Leaves are numerous, 6.3 to 9.0 cm long, with broad rounded lobes at the base, dark green and glabrous above, pale and dull beneath. Fruits occur in a solitary, pedunculate, fleshy spike 2.5 to 3.5 cm long, 5 mm thick, ovoid, oblong, erect, blunt, blackish green in colour and shiny. Odour is aromatic and taste is pungent. Minute achenes are closely packed in the fleshy axis of spikes. Male flowers are naked and bear two short stamens. Female flowers too lack perianth and bear a short carpel consisting of very short style with three to four lobed stigma and unilocular ovary. The male and female plants are morphologically very similar till the formation of spikes, and differ in the morphology of their reproductive spikes. Male spikes are longer (2.5 - 7.5 cm) and slender (Fig. 6) than the shorter (1.5 - 2.5 cm) and thicker (0.5- 0.7cm) female spikes (Fig. 5). Long pepper has high demand for its medicinal properties and for its use in ayurvedic and traditional system of medicine (Oommen et al., 2000; Banerjee et al., 1999). It is cultivated in many places including forest clearings, and flowers once a year especially during the rainy season. Among the various plant parts, mature spikes of female plants, known as long pepper, fetch high market value for their superior therapeutic properties and utility in the treatment of
Fig. 1. Dried Long pepper.
Fig. 2. *Piper longum* plant with spikes.
Fig. 3. South East Asian Long pepper plant with ripe fruits (*Piper retrofractum*).
Fig. 4. South Asian Long pepper in flowering (*P. longum*).
Fig. 5. *P. longum* female spike.
Fig. 6. *P. longum* male spike.
respiratory tract diseases and as a spicy culinary ingredient (Viswanathan, 1995). The plant is commonly found in Magadha and Vindhya, both in present day Bihar, growing near streams or other water resources as creepers. The plant, considered indigenous to the hotter parts of India, is found growing wild on the west coast as an undergrowth in the evergreen forests of the Western Ghats and it is also cultivated (Sivarajan and Indira, 1994). It occurs in the hotter parts of India, from Central Himalayas to Assam, Khasi and the Mika hills; the lower hills of Bengal; and the evergreen forests of the Western Ghats from Konkan to Travancore. Long pepper is native to Northeast India. Globally, the species is distributed in the Indo-Malaysian region, Sri Lanka, and a few species from Nicobar Island (Henry et al., 1987; Commen et al., 2000). The threat status of this species has been assessed as endangered in Tamil Nadu (Arv. Dr. and in Kerala as near threatened (Ravikumar and Ved, 2000).

**Adulteration in Piper longum**

Certain rare and expensive medicinal plant species are often adulterated or substituted by morphologically similar and easily available or less expensive species. In Asia, two different *Piper*—with exactly the same sensoric properties—are used in traditional and Ayurvedic medicine: South East Asian long pepper, *P. retrofractum* and South Asian (Bihar) parippu (species) *P. longum*. *Piper retrofractum* (Fig. 3) from Indonesia has rods a little bit smaller than *Piper longum* (Fig. 4) from India (Bengal pepper), which is mostly cultivated in Indonesia and Thailand. These species are often not clearly distinguished in the spice trade. In western countries mostly the latter is available. There is mention of four types of Pippali in Rajaangaharini (Sharma, 1983; Sivarajan and Indira, 1994) namely: Pippali, Vanaprasth, Shishunala and Gaajapippali. Sharma (1983) equated the former three with *Piper longum*, *P. syringatum* and *P. retrofractum*, respectively. However, physicians do not make any distinction between the three and *P. longum* is accepted for all. Gaajapippali is considered as a different drug but its identity is highly controversial. According to some, gaajapippali is the fruits
of *Piper nigrum*—a species under cultivation in India and Malaya (Churukker, 1982; Sharma, 1985). Physicians in Kerala equate this species with a different drug, namely chavap. Yet, others have associated the spices of *Schizandra officinalis* of Araceae as gajappal (Malaya, 1956, Nadkarni, 1956; Chopra et al., 1956; Moser, 1980). However, a market survey has revealed that *Bakambeeck sandwense* (Balanophoraceae), a root-parasite, which superficially resembles the inflorescence of *Schizandra officinalis* and chopped stem of *Bakambeeck sandwense* (Araceae) are also used as gajappal (Sivarajan and Indira, 1994). Hence, the present study has significance with reference to detecting adulteration in long pepper spikes.

Long pepper probably went to Europe before the now dominant black pepper. It was highly priced during the time of Roman Empire, fetching about three times the price of black pepper. With its taste pungent and sweet at the same time, it was perfect for Roman cookery, since Romans were especially fond of these two taste sensations. In olden days, long pepper was nearly unknown and sometimes hard to obtain. Since terpene components are missing in its aroma, long pepper cannot be substituted by ordinary black pepper. Its hot and sweet taste goes well with spicy cheese specialties or white sauces. Since long pepper is more pungent than black pepper, it must be used with care, unless one likes fiery food. In India, the main use of long pepper is in pickles.

Rather remarkably, long pepper is also renowned in popular parts of Africa, namely in the Islamic regions of North and East Africa, where was introduced by Arab traders. Long pepper is sometimes found in the complex spice mixtures of Morocco (Ras el Hanout), but it is also of some importance for the cuisine of Ethiopia, where long pepper is usually found in traditional meat stews (wat), mostly together with black pepper, nandet, cloves and turmeric. Spice usage in Ethiopia (and its neighbour Eritrea) furthermore parallels India: for example, the classical Ethiopian spice mixture *berbere* resembles Indian masalas not only in its list of ingredients, but also in its preparation process. *Berbere* is a very hot mixture and traditionally used to spice mutton dishes. It
is made by toasting dry chilies a few minutes until they darken and then adding long and black pepper, ginger, coriander fruits, fenugreek and a little bit of ajwan.

Cinnamon, cardamom seeds, cloves and even allspice achieve sweet tones which are essential for the cooking style of all Arabic nations. As *Piper longum* is excessively exploited from its natural resource, the species has now become very rare in the forests of Kerala (Nair, 2000).

**Cultivation Practices**

*Piper longum* is typical to tropical humid climate and prefers shady moist conditions, well-drained sandy soil of pH range 5.5 to 8.5 with rich humus. Latente soils with organic matter content and water holding capacity are suitable. Areas with good rainfall and high relative humidity are conducive for its successful growth. It is also grown as an inter-crop in coffee plantations in the plains, though altitude of 3000-5000 ft above sea level is recommended. The plant grows as a bushy runner. It is propagated by vegetative means, through cuttings, semi-hard stem cuttings 10 to 12 cm long with at least 3 nodes are planted in shaded nursery beds with the uppermost bud exposed. A spacing of at least 12 to 15 cm has to be provided between each pair of cuttings. The stem cuttings root in 10 to 15 days and success is 60 to 70%. It is also cultivated through tillers. The tillers arising from the base of a mature plant are separated and planted individually. The sprouted cuttings are planted in the field at an interspace of 2 x 2 feet in 30 cm pots. Though it is a shade loving plant, for better fruiting 50% shade is considered best (Commere et al., 2000).

**Traditional Medicinal Uses**

The herb has very pungent fruits, which are black in colour and are used with condiments while serving liquor (Aliy and Kollectal, 1966). The long pepper crushed is useful in the affections of eyes and in intermittent fever (Marikal, 2003). In traditional and ayurvedic medicines, mature spikes of female plants (long pepper), these
Stems, roots and leaves are extensively used in the treatment of bronchial diseases, dyspepsia, worms, anorexia and as a memory enhancing and aphrodisiac agent. Pippali is an important drug made from long pepper that is capable of improving intellect, memory power and also to regain health by dispelling diseases. It controls cough, ascites, leprosy, diabetes, piles, colic indigestion, anemia, dispels cardiac and splenic disorders, chronic fever, loss of appetite, worm troubles and it rehabilitates related "Vata" and "Kapha" (Vaswathan, 1995). Pippali on increasing dosage is effective in patients with respiratory disorders. It is also used as an antidote to snakebite and scorpion-sting (Chomman et al, 2000) but its versatility has not been scientifically validated. It is used as a carminative, sedative, emollient, and as general tonic. Long pepper enhances thermogenic response or release of metabolic heat energy and is used in the preparation of abhayarisam, dukkasthara, chyavanaprasham, pippladasaavam, in cold relief balm, pain balm and joint care balm. It is also used in medical preparations such as abana (heart care), bernisa, geri force (geri care/stress care), cough syrup, digstyon and chyavanamrutha (Swaranjan and Indira, 1994). In respiratory tract disorders long pepper is used as counter irritant and analgesic when applied locally for muscular pains and inflammation, as snuff in coma and drowsiness and as antihelmintic (Neelam and Krishnaswamy, 2000, 2003).

**Phytochemistry**

The long pepper fruits contain calcium 1220 mg, phosphorous 160 mg and iron 62.4 mg/100 g. Other nutrients have not been analyzed (Neelam and Krishnaswamy, 2000, 2003). The content of piperine (about 6%) is slightly higher than in black pepper. On the other hand, long pepper contains 1% volatile oil, resin, alkaloids piperine and piperlongumine, a waxy alkaloid N-subsutyl deca-trans-2-trans-4-decadiolide, sesquiterpene hydrocarbons and ethers (thujene, a-caryophyllene, b-caryophyllene oxide, each 10 to 20%, c-singiberene, 5%), saturated aliphatic hydrocarbons 18 % pentadecane, 7% tridecane, 6% heptadecane. Roots contain piperine, piperlongumine
on piparidine and dihydroxyferulic (Barret et al., 1985). Excessive use of Ayurvedic herbs may cause high pain and are often taken in combination with others to neutralize the toxicity of one herb with the opposing effect of other. However, it is not advisable to take excessive quantities of long pepper except under the supervision of a qualified professional (Neelam and Krishnaswamy, 2000).

**Pharmacological Activities**

A common use of the fruits is in the prevention of recurrent attacks of bronchial asthma. Although there is no convincing explanation for the extensive use of pepper varieties, there is unequivocal evidence for its beneficial effects. Clinical studies have revealed that pipard is very effective in the treatment of bronchial asthma in children (Dehandkar et al., 1984; Anshuman et al., 1984). Compounds isolated from long pepper were found to have antitubercular activity (Kumar et al., 1979) and to induce infertility in female rats (Kharakute et al., 1978; Maiterley, 1977). Long pepper spikes were used in the treatment of chronic malarias (Arundkar and Vaidya, 1983).

Dried capsicamaline from the dried fruits has displayed coronary vasodilating activity (Shah et al., 1986). Long pepper has been shown to be hepatoprotective as it has the ability to prevent carbon tetra-chloride-induced liver damage in rats. Along with *Piper sarmentosum* it has been useful in the treatment of viral hepatitis (Kaul and Kapil, 1995). Its activity against *Entamoeba* was demonstrated in rats (Ghoshal et al., 1986). Fruits used in traditional systems of medicine are effective against experimental infections of giardiasis in mice (Tripathi et al., 1999). Fruits of long pepper have also been shown to effectively reduce passive anaphylaxis in rats (Chatterjee, 1999). In view of the commercial, economic and medical importance of *Piper* sp., several workers have investigated the species pharmacologically, chemically and also pharmacologically (Neelam and Krishnaswamy, 2000). Protective action of piparidine against gastric ulcer was reported by Bai and Xu (2004). Dection of immature fruits and roots is used against chronic bronchitis, cough and colds (Coomen et al., 2000). The antibacterial
activity of the piper-ureidoglutaine isolated from long pepper against *Bacillus subtilis*, and the isolate piperine against *Staphylococcus aureus* was also reported (Selvivas Reddy et al., 2001).

**GERmplasm Conservation and Molecular Markers**

The tools of molecular biotechnology are being increasingly applied for characterization of plant diversity and they have a major role in assisting plant conservation programmes (Benson, 1989). Four main areas of biotechnology, which can directly assist plant conservation programmes, are: 1. Molecular marker technology; 2. Molecular diagnostics; 3. Tissue culture (in vitro propagation); 4. Cryopreservation.

Molecular marker techniques have a key role in enabling the assessment of plant diversity at the genomic level (Eshed et al., 1997; Karp et al., 1997). The elucidation of population structures and pattern of gene distribution within ecosystems provides information which can be used to support in situ conservation programmes. Assessment of RFLP that permit the detection of specific marker genes and polymerase chain reaction (PCR) based marker detection technologies have been used in association with RAPD analysis. Practical application of DNA marker technologies includes advising on germplasm collection missions and gene bank design. Importantly, knowledge of molecular genetic diversity can greatly assist the selection processes associated with ex situ conservation of germplasm and more directly facilitate plant germplasm collection and gene bank management. Molecular marker techniques also enable herbarium and germplasm curators to identify significant omissions in germplasm collections and thus enable them to target more effectively on the acquisition requirements of future germplasm conservation (Harris, 1999; Simpson, 1999).
DNA marker technologies have an important role in the monitoring of genetic stability in conserved germplasm. It is essential that storage methods can be used with confidence and molecular markers can be used to confirm that conserved germplasm retains genetic fidelity. This may be especially important for germplasm, which is conserved using tissue culture procedures. Tissue culture (or in vitro) technologies have had a major impact on the on-site conservation of plant genetic resources. Micropropagation, using somatic embryo and shoot tip culture techniques, assists many crop plant improvement programmes and increasingly these methods are being used for the conservation of endangered plant species (Mandal, 1999). Plants which are vegetatively propagated have particular conservation problems, as their seeds are not available for banking. In vitro conservation using tissue culture methods is the only reliable and long-term means of preservation (Anderson and Craft, 1997).

The development of molecular markers has led to intense investigation and characterization at the genetic level of plants with traditionally well-developed genetic systems. But the advantage provided by molecular markers over classical genetic marker systems, is the ability to tap and utilize information on naturally occurring polymorphisms within populations. There are three types of genetic markers in genomic analysis:

1. Morphological markers (based on shape, colour, size, and height).
2. Protein based markers (e.g. isozyme markers) and
3. DNA markers (Table 1).

Molecular markers generally refer to biochemical constituents, including primary and secondary metabolites and other macromolecules such as nucleic acids. Secondary metabolites as markers have been extensively used in quality control and standardization of botanical drugs. Variation among genotypes within a species is the raw material for genomic analysis. Most natural populations have relatively high levels of polymorphisms due to small changes in DNA sequences such as point mutations,
base substitutions, insertions, deletions and translocations, which are largely neutral. Recent molecular techniques allow the detection of these polymorphisms and have proven extremely efficient in the discrimination of individuals. Such polymorphisms and the molecular markers associated with them are inherited in Mendelian fashion. They are extremely abundant and found throughout the genome. In addition, developmental, tissue specific and environmental factors do not influence the detection of these polymorphisms, making them excellent genetic markers.

To be a genetic marker, the marker locus has to show experimentally detectable variation among the individuals in the test population. Polymorphism is defined as detectable and heritable variation at a locus. A genetic marker is operationally defined as a heritable polymorph with clear genetic interpretation and repeatability (Li, 1998). It is also important that the marker assay is repeatable at different times in the same or different laboratories. DNA markers have several advantages over typical phenotype markers. They are reliable for informative polymorphisms, as the genetic composition is unique for each species and is not affected by age, physiological conditions nor environmental factors (Henry, 1997; Zaykin et al., 2004).

A genetic map is an abstract model of the linear arrangement of a group of genes and markers. A "Gene" has been traditionally defined as Mendelian factor or a piece of DNA identified by a known factor or by means of biochemical assay. The marker is a cytological marker, a variant based on a change in a known gene or protein or a piece of DNA without a known function. Both the gene and the markers have simple inheritance that is followed through generations. A gene with known function is considered as a genetic marker if it contains detectable variation. The polymorphic markers are usually too variable to identify genome homology among different species, because the variation within the species confounds the variation between species. Linkage information obtained from genetic markers can follow different models of inheritance in the common mating systems of plants. F2 populations, where co-
dominant markers segregate in a 1:2:1 ratio and dominant markers in a 3:1 ratio are
often used in mixed plants (Liu, 1998).

Molecular markers can be considered as additional characters or traits for the
evaluation of genetic differences. Molecular data will help to identify differences in
genomic level, but these may not be used to determine issues such as the extent to
which the new variety is substantially derived from earlier varieties. The concept of
substantial derivation in the protection of plant varieties relates to traits of commercial
value and not to genetic differences that do not alter the performance or value of the
plant. The distinction of plant varieties may be based upon differences in the variance
of some characters rather than differences in their mean value. Molecular marker data
may be analyzed in the same way. DNA-based molecular markers have been widely
used for authentication of plant species of medicinal importance (Josh et al., 2004).
Variation between genomes can be studied at several levels. The analysis of
polymorphisms using randomly amplified polymorphic DNA (RAPD), microsatellites and micro
satellites provides a means to look at variations across the entire genome or at defined
loci and has many potential applications from population genetics to molecular
pathology. Molecular techniques provide useful tools for the study of the influence of
genetic diversity of plants on the sustainability of the ecosystem.

TYPES OF MOLECULAR MARKERS

Random Amplified Polymorphic DNA (RAPD)

The RAPD markers are generally assumed to be of nuclear origin, but that may not
always be the case. In a normal PCR reaction two distinct oligonucleotide primer of
known sequence flanking a region of interest are used to synthesize the DNA fragment
between the two primers. Repeated synthesis of the fragment leads to an amplified
number of copies of the specific fragments bounded by the oligonucleotides. In a
RAPD reaction a single ten-nucleotide sequence chosen at random has been used as a
primer. The amplification occurs at the generic region flanked by inverted repeats of the primer. Amplified PCR fragments are visualized under ultraviolet light following separation on an agarose gel and staining with ethidium bromide. In the case of RAPDs, the polymorphisms detected in different individuals are due to mutation or changes at DNA sequence either in the regions homologous to the oligonucleotide or in the surrounding sequences, similar to the situations for RFLPs. These changes may inhibit oligonucleotide hybridization causing the PCR reaction to fail so that no amplified fragment is produced, or it may cause the size of the fragment to change. Polymorphisms are detected as presence or absence of bands and although in theory heterozygote should give lower intensity bands, very small changes in reaction conditions or DNA concentration can mask this effect; therefore, it is usually not possible to distinguish heterozygote (Henry, 1997).

RAPD is a widely applied technique for characterization of DNA from plants and other organisms (Reilly et al., 1997) using PCR with short oligonucleotide primers or arbitrary (random) sequence to generate genetic markers. This has been the basis of the RAPD method (Williams et al., 1990), arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990) and DAF (DNA amplification fingerprinting) (Caetano-Anolles et al., 1991). The main issue associated with the use of these techniques is the problem of ensuring reproducibility of amplification profiles. The nature of the amplification process with short primers is such that many sites in the genome are potential templates and the profile obtained may be influenced by any variation in the method used to prepare the DNA template, the exact reaction composition and conditions used in the PCR. This means that variation in the concentration of primer or template can result in the amplification of different products (Viceralidze and Wakeland, 1995). Standard primer, nucleotide and magnesium concentrations, exact reproduction of temperature cycling conditions, DNA polymerase type and activity are essential for reproducibility. Both the quantity and the quality of the template DNA preparation have the potential to substantially influence the final result.
**Table 1: Molecular Methods Suitable for Different Levels of Genetic Distinction**

<table>
<thead>
<tr>
<th>Type of marker</th>
<th>Individuals</th>
<th>Variety</th>
<th>Species</th>
<th>Genus</th>
<th>Family</th>
<th>Higher levels</th>
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<tbody>
<tr>
<td>ESR</td>
<td>X</td>
<td>X XXX</td>
<td>X</td>
<td>X</td>
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<tr>
<td>RAPD</td>
<td>X</td>
<td>X XXX</td>
<td>X</td>
<td>X</td>
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<tr>
<td>RFLP</td>
<td>X</td>
<td>X XXX</td>
<td>X</td>
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<td></td>
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<tr>
<td>Nuclear genes</td>
<td>X</td>
<td>X XXX</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
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<tr>
<td>Mitochondrial genes</td>
<td>X</td>
<td>X XX</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
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<tr>
<td>Chloroplast genes</td>
<td>X</td>
<td>X XX</td>
<td>X</td>
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<tr>
<td><strong>Sequencing</strong></td>
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<tr>
<td>Internal transcribed spacer</td>
<td>X</td>
<td>X XX</td>
<td>X</td>
<td>X</td>
<td></td>
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<tr>
<td>Riboosomal genes (ISSR)</td>
<td>X</td>
<td>X XX</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
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<tr>
<td>Chloroplast genes</td>
<td>X</td>
<td>X XX</td>
<td>X</td>
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<tr>
<td>UPOs (conserved proteins)</td>
<td>X</td>
<td>X XX</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
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<tr>
<td>Conserved proteins</td>
<td>X</td>
<td>X XX</td>
<td>X</td>
<td>X</td>
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</tbody>
</table>

*Number of X indicates increasing use of the method (Henry, 1997)*

RAPD marker analysis has been widely used in many crop species, as they are relatively cheap, simple to use and the only major instrument needed is a thermocycler. Commercially available oligonucleotide primers can be used directly to screen for polymorphisms. The major criticisms of random amplification methods are the low reproducibility between different laboratories since the reaction is extremely susceptible to changes in buffer composition, DNA concentration and even different models of thermocyclers. RAPD markers are not necessarily associated with coding sequences. This is advantageous when coverage of the whole genome including repetitive and non-coding sequences is required. RAPD and RFLP markers were shown to separate 106 cacao genotypes (NVGoran et al., 1994). Many reports of gene tagging, mapping and characterization of germplasm using RAPD markers continue to be published with the normal precautions necessary for all PCR reactions, which are useful and considered as efficient markers (Henry, 1997).
Restriction Fragment Length Polymorphism (RFLP)

RFLPs are codominant and multi-allelic and may often be used as gene specific probes. In practice, RFLP probes commonly recognize small gene families and one probe can provide several segregating markers. PCR based markers are frequently dominant and each primer may generate several bands. It may be difficult to determine which segregating bands in different families correspond to the same locus. It is based upon hybridization of a probe to probe is a specific DNA sequence designed to hybridize with and thus detect a target sequence or sequences in the unknown sample to fragments of genomic DNA following digestion with restriction enzymes. Differences in the sequence at or around the sequence with which the probe hybridizes may result in polymorphisms in the length of the fragments detected by the probe. The first DNA based molecular markers were developed to detect polymorphisms produced by changes in the distance between two restriction enzyme sites (Botstein et al., 1980).

These polymorphisms were caused by mutations in or around the restriction sites. If one homolog suffers a point mutation (nucleotide substitution or deletion) at one of the restriction enzyme sites, the restriction enzyme will no longer recognize the sequence at that location and will not cut the DNA. Obviously a single restriction digest of plant DNA would produce a smear of bands, in which changes in individual fragments would be impossible to detect; therefore the Southern blot technique is used to detect specific restriction fragments. Single copy genomic DNA clones or cDNA clones are used as probes for specific regions of the genome. RFLP technique was applied for characterization of *Capsicum* used for interspecific genetic variation within the genus *Capsicum* and also for DNA fingerprinting of pepper cultivars (Joshi et al., 2004).
Sequence Tagged Sites (STS) and Sequence Characterized Amplified Region (SCAR) Markers

Several strategies to develop different types of molecular markers useful for tagging resistance genes have been described. Among these methods, bulked segregant analysis (BSA; Michelmore et al., 1991) combined with several types of molecular markers have been extensively used to find markers linked to genes of interest. Sequence tagged site is a short unique fragment of DNA (~300 bp). The polymorphic STS markers are also commonly used for genetic analysis of plants. STS may be based upon any known sequence in the genome (Reaman and Jung, 2000). The transformation of these markers into more stable and easily applicable markers, such as SCARS (Paran and Michelmore, 1993) or cleaved amplified polymorphic sequences (CAPS) (Korthazy and Ausbel, 1995) is generally the next step before their routine application in marker assisted selection programmes (Babu et al., 2004).

Sequence characterized amplified regions (SCARs) are PCR based markers that represent a single genetically defined loci that are identified by PCR amplification of genomic DNA with a pair of specific primers. Paran and Michelmore (1993) succeeded in producing SCARs in lettuce. The sequence of the amplified product is used to design longer primers that offer greater specificity. A SCAR may be applied to routine screening with sample DNA preparations without the restrictions that might have applied to obtain reliable data from the RAPD analysis on which it was based. The polymorphism revealed by RAPD markers as the presence or absence of specific band amplified by PCR, which are dominant, can be converted into codominant SCAR markers (Fig 7). Such markers are considered to be more reliable than RAPD markers (Hickey, 1988).
Amplified Fragment Length Polymorphism (AFLP)

AFLP technique is used to visualize DNA polymorphisms between samples, to identify a specific DNA sample relatedness. It is a source for genetic marker to generate linkage maps or to identify molecular markers linked to phenotypic traits or genetic loci. It combines both classical restriction-based and recent PCR-based approaches. Genetic variation within Brachypodium distachyon cultivars has been studied using AFLP markers, and sex-linked AFLP markers were developed in Cannastra sativa (Petli et al., 2005) and Sesame (Sabino et al., 2002). AFLP is a powerful, reliable, stable and rapid assay with potential application in genome mapping, DNA fingerprinting and marker-assisted breeding (Maheswaran, 2004; Jothi et al., 2004).

ISSRs, SSR and Mini/Microsatellite Markers

Intragenic simple sequence repeats (ISSRs) polymorphism is a specific primer-based polymorphism detection system, where a terminally anchored primer specific to a particular simple sequence repeat (SSR) is used to amplify the DNA between two opposed SSRs of the same type. Polymorphism occurs whenever any genome is
missing in one of the SSRs or has a deletion or insertion that modifies the distance between the repeats (Kotha et al., 2004). In hemp sex-linked simple sequence repeat (SSR) markers are described (Redd, 2005). Genetic diversity analysis of elite pearl millet inbred lines investigated using RAPD and SSR markers have been reported (Charitrasadhipta, et al., 2005). Mini satellites are tandem repeats of sequence ranging from 6 to 100 bp in the genome. Mini satellites are also referred to variable number tandem repeats (VNTR), which can be detected using either hybridization or PCR approaches. Genomic DNA can be digested using restriction enzymes that recognize restriction sites flanking the tandem repeat. The cutting yields fragments containing one or more of the repeats with different lengths that can be detected using a probe designed from DNA sequence flanking the repeats or from the repeat itself. VNTR can also be detected by amplifying the segment containing different number of repeats by using primers flanking the segments for PCR. The polymorphic bands result from the variation in the number of the tandem repeats (Matheswaran, 2004).

**Single Strand Conformational Polymorphism (SSCP)**

SSCP can detect DNA sequence alterations as small as a single nucleotide change. Electrophoretic mobility of single stranded DNA in non-denaturing polyacrylamide gels depends on size and sequence characteristics. This method exploits the tendency of single-stranded DNA to form intra-molecular base-pair, resulting in a sequence dependent conformation with a specific mobility in acrylamide gels. Changes in DNA sequence, even in a single base pair can cause alterations in the conformation and result in changes in electrophoretic mobility. In practice, SSCP can be detected using the following two methods. The DNA can be cut with restriction enzymes then run on a gel to separate by conformation. A southern blot of the gel is then done and a specific fragment is used as a probe for hybridization. The other method uses PCR to amplify a specific fragment, which is then run on a conformational (high resolution acrylamide) gel (Liu, 1998).
DNA POLYMORPHISM AND MARKERS

The polymerase chain reaction (Saiki et al., 1988) has been the basis of a growing range of newer techniques. The PCR-based tests generally have advantages in speed and sensitivity but in some cases hybridization-based tests may be cheaper to conduct. Molecular marker techniques of all kinds may be used to define identity, purity and stability of germplasm. The range of techniques available for immediate application varies with major species offering a wide range of established molecular markers, and minor or less well-studied species require evaluation of potential marker systems. Arbitrary marker techniques such as RAPD analysis are attractive for the less well-known species because they can be applied without prior knowledge of gene sequences and in the absence of any genetic map or prior molecular studies. A useful method for the identification of plant varieties for the purpose of commercially protecting them should be based if possible on methods that can be reliably reproduced in any laboratory. Genetic variation in plant populations may be caused and maintained by a variety of mechanisms including mutation, sexual recombination, migration and gene flow, genetic drift and genetic selection (Joshi et al., 2004).

The need to assess genetic stability is related to the method of propagation of the plant. Vegetatively propagated species should show very little variation between generations. Tissue culture may introduce some genetic changes and sexual reproduction can be the basis of very large differences. Again, the application of molecular techniques to the assessment of genetic stability does not differ in principle from the assessment of other non-molecular traits. The main requirements of any molecular test to be used in plant genetic protection are that:

1. The test may be repeatable, both within and between laboratories
2. They may be objectively assessed or scored
3. They provide satisfactory discrimination between varieties (Smith and Chin, 1992).
Genetic Diversity Analysis

Population genetics focuses on the frequency distribution and origin of genes in populations. It combines Darwin's evolutionary theory with Mendelian genetics and molecular biology to quantify the evolutionary process. At the population level, genetics can be characterized by allelic and genotypic frequencies. Forces changing the allele and genotype frequencies are mutation, natural and artificial selection, population admixture (migration), and random genetic drift. Population genetics has contributed significantly to the understanding of evolution (Mit, 1998). The three main application areas for polymerase chain reaction (PCR) are:

1. The identification of genotypes
2. The assessment of genetic diversity and/or relatedness
3. Segregation and linkage analysis for genetic mapping

Once fingerprint patterns have been generated and scorable bands are assigned to specific positions in all lanes to be compared, different strategies may be followed to quantify the pairwise similarity of the genotypes represented in the different lanes. A phylogenetic pattern forms a branching tree, which represents genetic similarity and genetic distance (Mit, 1998).

Random Amplified Polymorphic DNA for Genetic Distance Studies

The type of molecular method used to measure genetic distances in plants will vary depending upon the magnitude of the genetic differences being assessed. The amount of variation detected suggests that this approach is likely to be most useful for analysis at the species or lower levels or at least the sub-generic level. RAPD markers have been used to analyze genetic relationships in taxa (Abe-elwafa et al., 1985). RAPD analysis may be useful for distinguishing different genotypes within a plant cultivar while sequence analysis of the ribosomal genes may allow species for higher-level analysis. Phylogenetic studies and analysis of population genetics both require careful data.
interpretation. All methods require certain basic assumptions and have strengths and weaknesses (West and Faith, 1990). Lactarius volemus, ssp. ornatus was indicated as the most likely wild progenitor for lentils. The variation within cultivated lentil was lower than that in wild relatives. RAPD has also been applied to the analysis of cocoa (Theobroma cacao) populations, revealing greater variation between populations than within a population (Russel et al., 1995). Mathematical methods have been developed to allow correction for the errors in estimation of genetic distance associated with the scoring of complex gels such as those generated in RAPD analysis and with the difficulty of reproducing the DNA extraction and amplification (Lambey, 1994).

The development of the PCR fingerprinting techniques, RAPD (Welsh and McClelland, 1990), has accelerated the detection of markers in plant genomes. By combining RAPD with bulk-segregation analysis, where two pooled DNA samples are formed from plants which have similar genetic backgrounds but differ in one particular trait, markers linked to that trait can be identified (Pena and Michelmore, 1995). The technique of RAPD offers a broad range of applications in the investigation of plant genomes. A promising prospect is the use of RAPD products as genetic markers. Male sterile and male fertile sugar beets were subjected to RAPD analysis with 6 different primers. Total, nuclear, mitochondrial and chloroplast DNA from each line was investigated. Reproducible DNA fingerprints were obtained from both organelar DNAs. Differences in band patterns of mitochondrial DNA between cytoplasmic male sterile and fertile lines were observed with 5 out of 6 primers, whereas chloroplast DNA patterns were generated by one of the primers. Thus, RAPD technique has been used to discriminate between different cytoplasms. Clear evidence was provided for the organelar origin of fragments in genomic RAPD patterns (Lorenc et al., 1994).

RAPD markers for the certification of F1 hybrids of Carola have also been reported. Stable DNA fragments that were homozygous and specific to the male inbreds were used to certify F1 hybrid populations in carola (Marshall et al., 1995). Preliminary
Linkage maps of cassava increase the efficiency of introgression of specific chromosomes or chromosome fragments from exotic cassava germplasm into various genetic backgrounds. Localization of genes of interest in cassava improvement schemes has been drawn from RFLP segregation data of single dose polymorphisms of RFLP and RAPD markers (Fregene et al., 1994). In *P. peruvianum* three DNA-based marker types were evaluated for linkage map construction: RFLPs were detected by Southern blot hybridization. STSs were detected by a combination of PCR, RFLP analysis and RAPDs. The applicability of STSs and RAPDs outside the mapping pedigree has been investigated to show that these PCR-based marker-systems are well suited to breeding designs involving interspecific hybridization (Bradshaw et al., 1994). RAPD markers for genes conferring resistance to mosaic virus were identified by screening near isogenic lines of *Geospermum excisum*. The genes introduced from the wild relative of tomato (*Solanum pseudocapsicum*) were located on the long arm of chromosome 9 (Colman et al., 1995). A diagnostic test to confirm interspecific *A. hypogaea* hybrids using RAPD from crude leaf DNA extracts has been reported by Datta et al. (1996). RAPD markers tightly linked to gall midge resistance in rice have been identified by Murakoshi et al. (1996). RAPD analysis for genetic variation studies (Buishiya et al., 2000) of aromatic and non-aromatic rice was done, also in *Piper* species (Chaiwattana, 2002).

**Marker Screening for Polymorphism**

Genome map construction is based on genome variation at locations that can be identified by molecular assay or traditional trait observations. Screening and identifying polymorphic genetic markers is the first step in an efficient experiment on genetic diversity studies. This screening is usually done by assayng a large number of possible genetic markers, such as PCR and RFLP assays, for a small set of progeny randomly sampled from the mapping population. When screening for polymorphic markers, false positives are rare, because a monomorphism marker cannot produce segregating genotype if the genotypes are determined accurately (Henry, 1997).
MOLECULAR MARKERS IN GERMPLASM ASSESSMENT

Various types of DNA-based molecular techniques are utilized to evaluate DNA polymorphism. There are hybridization-based methods, polymerase chain reaction (PCR)-based methods and sequencing-based methods (Bustein et al., 1989; Joshi et al., 2004). DNA markers are typically a small region of single sequence polymorphism in different individuals within species. The fragment can be detected by nucleic acid hybridization, which uses another fragment from the same locus, which has been isolated and purified from the same or related locus of the same or related species. This is the formalism for RFLP markers; another approach is based on the amplification of sequence using PCR. Two primers (flanking the target sequence) designed using a known sequence of the segment are needed. Microsatellites, sequence tagged sites (STSS), ESTS, etc., have been commonly used as genetic markers based on sequence specific PCR. Shorter, arbitrarily chosen primers have also been used to amplify random polymorphic DNA markers (RAPD & AFLP).

Bulk Segregant Analysis (BSA)

BSA has been widely used as a tool to find markers linked to target genes or to other markers. In BSA, the DNA samples from different individual progeny in a family are pooled into a bulk sample by genotype class or by phenotypic class. A polymorphic marker that shows a clear difference between the two bulks is likely to be linked to the target gene or nearby markers. PCR based markers have been commonly used in BSA. For RAPD markers the principle behind BSA is that the low frequency allele will not be amplified (Michelmore et al., 1991). In rice, bulked segregant analysis was employed in conjunction with RAPD technique from hybrids. Survey of 389 open pollinated progeny revealed five RAPD fragments linked to the sterility gene, of which four were sterility linked. Use of probe revealed polymorphism in southern analysis. Based on chromosome mapping, sterility markers were used for selection in rice (Subudhi et al., 1996). RAPD analysis has been carried out to determine the molecular
relatedness among Himalayan yew-Taxus wallichiana (Subba et al., 2006). A sequence-tagged site (STS) marker identified by RAPD analysis is reported to be linked to a leaf rust resistance gene in wheat. Out of 80 random primers tested, one RAPD band was selected, as the additional band present in the resistant lines was cloned, sequenced and STS primer specific for rust resistant gene were designed (Nair et al., 1998). A single major gene flanked by eight RAPD markers and co-segregating with the yellow seed coat colour trait was identified in Brassica napus (Sarwar et al., 2001; Zhu-wen et al., 2005). In principle on the basis of RAPD amplification products, genetic similarity was estimated in spined and spineless types of regenerants (Sonaji et al., 2002) using similarity coefficients.

RAPD based molecular markers have been found to be useful in differentiating different accessions of Taxus wallichiana, Neem, Junipera communis, Cocos nucifera, Phoenix, Alnus rhombifolia and Andropogon paniculatus collected from different geographical regions (Joshi et al., 2004); micropropagated plants of Robinia pseudoacacia (Karanvir, 2002) and vegetatively propagated plants of Piper betle (Ramade et al., 2004).

Arbitrary primer based RAPD was used as genetic marker for species identification in millets (Singh et al., 2004), in grasses (Latha et al., 2004) and in Solanum (Singh et al., 2006). Studies were conducted in cotton using RAPD and RFLP marker (Vafaei-Tabari et al., 2004; Rana & Bhat, 2004), in basmati rice varieties using microsatellite markers (Sowah et al., 2004), in rice (Wang et al., 1995; Zhang, 1994) for male sterility gene. SCAR markers were also developed for blast resistance (Nagao & Chattoo, 1996) and in pea using RAPD and SSR markers (Chandra-shakara et al., 2007). Genetic diversity analysis was carried out in baga (Cirsium burmanni) (Britte et al., 2006), coconut (Manickam and Nagaratnam, 2007) and Brassica (Kalita et al., 2007) using SSR markers and in eggplant using isozyme markers (Manjeet kaur et al., 2004).
Information on the DNA sequence is needed for many of the markers described such as microsatellites, STSs, and polymorphic ESTs. A single arbitrarily chosen short oligonucleotide can be used as a primer to amplify genome segments flanked by two complementary primer-binding sites in an inverted orientation. Short primers with an arbitrary sequence can be complementary to a number of sites that occur on opposite strands of a segment of DNA in an inverted orientation. If the distance between the sites is short enough for PCR, then the segment flanked by the sites can be amplified. Amplification on different segments is independent. If polymorphism exists in the binding site among different genotypes or if the fragment length differs at the same site from genotype to genotype, then a RAPD marker is obtained. Only a small amount of DNA from each genotype is needed as templates.

A set of primers is generally screened for reproducible polymorphisms. Different primers may identify different polymorphisms and have different reproducibility. Different alleles at the same locus are generally distinguished by the presence or absence of a band of a particular size. The presence of a band indicates that the phenotype is dominant to that of its absence. The band is present if the genotypes are homozygous or heterozygous for the locus that is amplified. The band is absent if the genotype is homozygous for a lack of site to amplify that specific fragment. A major advantage of RAPD markers is that it is easy to obtain large numbers of markers and the most informative markers can be selected. The expected number of amplified products using an arbitrary primer is a function of genome length, number of nucleotides in the primer and the maximum fragment length that can be amplified (Li, 1998c).

Assuming that nucleotide distribution is random and that the primer is arbitrarily designed and complete complementation occurs between the primer and the binding sites in the DNA templates, then the expected number of amplified products is:

$$b = 2/N^{1/2}$$

$b$ = maximum length of amplified fragment in bp
$N$ = genome size in bp
$m$ = number of nucleotides in the primer.
For a single strand of DNA, the expected number of binding sites within a genome with $N_{bp}$ for a primer with a nucleotide is $N_{bp}/4$. The number is doubled for two strands of DNA. Since a single primer may generate several polymorphic markers, screening a large number of primers on a small number of genotypes in a mapping population is a useful method to obtain a large group of markers having high information content (Lu, 1998). DNA sequence variation among different genotypes within a species is the foundation for genetic analysis. The genetic co-linearity among different species is the basis for comparative mapping. Similarity is greater between genomes of closely related species, while distantly related species show greater divergence in genomic structure.

The relative information content of molecular markers can be compared by calculation of polymorphism information content (PIC) (Anderson et al., 1992). This index is used to compare the value of two different marker systems in the analysis of genetic polymorphism. The maximum PIC value for a RAPD marker would be 0.5 (band present in 50% of individuals; since only two alleles are assumed in analysis of RAPDs, presence and absence of the band). The choice of a particular type of molecular marker may be determined by many factors in addition to the PIC value of the marker. Estimation of similarities based upon RAPD results for a set of samples A & B can be calculated as follows:

\[
\text{Similarity (S)} = 2 \left( \frac{m}{n x y} \right) \left( \frac{n x + n y}{n x y} \right)
\]

- $n x y =$ number of bands in common to sample A & B
- $n x =$ number of bands for sample A
- $n y =$ number of bands for sample B

Jaccard’s coefficient is calculated as $I = \frac{m x y}{m x + m y - m x y}$ (By Nei and Li, 1979),

- $m x y =$ number of bands common to A & B,
- $m x =$ total number of bands present in all samples,
- $m y =$ number of bands not present in A or B but found in other samples.

Frequency of an allele $I$ in the population is defined as the probability that a haplotype (a particular haplotype combination of alleles in a defined region of a chromosome or a
gerontology carries the allele A. Allele frequency is distributed as a genomic series. Heterozygosity of a locus is defined as the probability that an individual is heterozygous for the locus in a population. For a genetic marker, a locus with heterozygosity higher than 50% is commonly considered a highly polymorphic marker (Liu, 1998).

It has been well documented that the geographical conditions affect the active constituents of medicinal plants and hence their active profiles. Many have studied geographical variation at the genetic level. Estimates of genetic diversity are also important in designing crop improvement programmes for management of germplasm and evolving conservation strategies.

The genetic variation in plant population is of considerable practical interest. Agriculture and food production depend upon the use of highly productive plant genotypes. The conventional breeding of crop plants is based upon selecting desirable genotypes from the genetic variants available and manipulating all or as many as possible of the desirable traits into one individual to develop a commercial variety. The diversity of species in an environment has been shown to contribute to the sustainability and productivity of the ecosystem (Tilman, et al., 1996). The diversity may be analyzed at many levels. Bio-diversity in ecosystem is usually considered in terms of the number of species; however, diversity within a species may also contribute significantly to the productivity of the system. Molecular methods offer objective options for assessment of biodiversity and may be the key to the development of appropriate conservation strategies (Szmidt, 1994).

Simple base substitution by insertion or deletion of a nucleotide may alter amino acids in a protein or may be silent depending on the position within the codon. The insertion or deletion of a single base can cause a frame shift; result in a major change in the protein structure and the resultant phenotype. The analysis of genetic variation or diversity in plants has been traditionally assessed by analysis of morphological or biochemical traits.
The assessment of phenotype may not be a reliable measure of genetic difference because of the influence of the environment on gene expression. The analysis of plant DNA allows the direct assessment of variation in genotype. Regarding the genetic identity and diversity of different cultivars and wild populations of *Piper longum*, very limited information is available at the molecular level. *Piper* is considered as a model genus for studies of evolution, chemical ecology, and trophic interactions (Dyer and Palmer, 2004).

**DIOECIOUS PLANTS**

Higher plants bear perfect flowers with both male and female organs. In certain plants, unisexual flowers of both sexes are found on the same plants (monoeccious) or on different plants (dioecious). Dioecy, a condition whereby the male and female flowers are borne on separate plants, is one of the most striking examples of evolutionary specialization. Approximately 5% of the genera of higher plants are dioecious. It has been argued that such extreme specialization of sexes into separate plants may have evolved as a mechanism to ensure out breeding (Darwin, 1877; Baras, 1980) or as an optimal allocation of resources between the sexes (Baras, 1980; Sæbø and Sæbø, 1997). Unisexuality promotes out breeding, which has adaptive advantages because it provides for genetic variability and exchange. The highest out breeding rates occur among dioecious plants because out breeding is obligatory, but rates can be variable in monoeccious plants in which out breeding is not mandatory (Dellaporta and Calderon-Urrea, 1999). Although only about 10% of plants are known to be strictly monoeccious or dioecious, unisexuality arose many times during plant evolution and is found in 55% of plant families. Because unisexuality evolves independently many times, there is no consistent genetic basis for sex determination in plants. In fact, many of the mechanisms found in animals are found in plants such as X to autosome ratios, active Y and autosome-determined systems (Farbos et al., 1999; Frasal and Bedhuarme, 2006).
A number of important crops, such as nutmeg, pistachio, cannabis, date palm and *Asparagus* are dioecious. In all these crops, a major problem for farmers is to identify the sex of saplings at an early stage so that they can cultivate in their orchards a sufficiently large number of productive female trees with only a minimal number of male trees. However, attempts to identify the sex of the dioecious species at an early stage have remained frustratingly unsuccessful. Efforts were made to understand the genetic basis of sex determination in plants and to develop methods to identify sex at an early stage employing molecular marker tools (Mulcahy *et al*., 1992; Hormaza *et al*., 1994; Biffi *et al*., 1995).

Most flowering plants are hermaphroditic, having flowers with both male and female parts. Many of the dioecious species of plants have chromosome-mediated sex determination. The taxonomic distribution of separate sexes and chromosomal sex determination systems in the flowering plants indicates that plant sex chromosomes have evolved recently through replicated independent events contrasting with the ancient origins of mammalian and insect sex chromosomes. Plant sex chromosomes, therefore, offer opportunities to study the most interesting early stages of the evolution of sex chromosomes. White campion (*Melandrium album*) is a dioecious plant with heteromorphic X and Y sex chromosomes. In male campion plants, a filamentous structure replaces the pistil, while in female plants the stamens degenerate early in flower development (Farbos *et al*., 1999).

The reproductive systems that pattern floral and sexual differentiation can be monomorphic, with a single bisexual flower type, or polymorphic, with two or more flower types (Irish and Nelson, 1989). Polymorphic reproductive schemes, including dioecism, monoecism and other variations are estimated to appear in about 7% of dicot genera and 6% of monocot genera (Yampolsky and Yampolsky, 1922). Dioecious and
monoecious plants develop unisexual flowers and thus possess at least two schemes for floral development within each species. In dioecious species, such as *Amaranthus* and *Mecardonia*, plants are either male or female, and most species have either staminate or pistillate flowers and produce either male or female gametes with clonal out crossing (Durand, 1965; Lazaro and Faller, 1970).

The ratio of males and females in a population of a dioecious species is generally based on genetic segregation of alleles at one or more loci. In some dioecious species such as *Mecardonia*, sex chromosomes have been identified cytogenetically, with the male being generally the heterogametic (XY) sex and the female the homogametic (XX) sex (Bittaker, 1923; Winge, 1953). In other cases, the sex of an individual is determined at fertilization. Studies with the dioecious *Mecardonia* plants suggest that genes controlling development of individual as male or female may cause sex expression by setting extreme endogenous levels of auxin and cytokinin regulators. The majority of flowering plants produce flowers that are perfect. In a small number of species, there is a separation of the sexual organs either monoecy, where the male and female organs are carried on separate flowers on the same plant, or dioecy, where male and female flowers are carried on separate male (staminate) or female (pistillate) individuals. The point of divergence from the hermaphroditic pattern exhibits wide variation between species, implying that the genetic bases are very different (Ariasworth et al., 1995).

**SEX DETERMINATION IN PLANTS**

Studies on sex determination in many species suggest that a variety of distinct strategies may be employed to produce unisexual flowers. In general, most studies support the model of alternate suppressible pathways for male and female differentiation (Durand and Durand, 1989). *Amaranthus* is dioecious with distinguishable sex chromosomes (Lopatin, 1979). XY and XYY individuals produce staminate
Flowers: XX individuals produce pistillate flowers (Franken, 1972; Lazarte and Palser, 1979). Under some conditions, XY individuals are andromonoecious, producing staminate and bisexual flowers. A series of genetic studies has suggested that genes controlling the sexual characters of individuals segregate as a dominant female suppressor and a dominant male activator at the Y chromosome. In the diploid dioecious species *M. matsumura*, experimental variation of XY and autosome dosage showed that the presence or absence of Y-chromosomes is the primary determinant of maleness or femaleness, while the number of X-chromosomes or autosomes present modifies the expressed maleness (Westergaard, 1958; Fränkel and Galun, 1972).

In *Silene latifolia*, a gene encoding a male-specific protein is linked to the X-chromosome, and it has a degenerate homologue in the non-pairing region of the Y chromosome. The Y-linked locus has degenerated because of nucleotide deletions and the accumulation of repetitive sequences. This discovery is both the first X-linked gene and the first pair of homologous sex-linked loci to be found in plants. The homology between the active X-linked loci and the degenerate Y-linked locus supports a common ancestry for these two loci (Guttman et al., 1998; Matsumura and Kawano, 2001).

In many dioecious plants, gender influences economic value, breeding schemes, and opportunities for commercial use of genetically transformed materials. In *Rumex americana* (Wood Sorrel) and *Silene* the sex of the individual plant is influenced by a dosage compensation mechanism based on the X chromosome to autosome ratio (Ainsworth et al., 1985; Matsumura et al., 1996). Yet the influence of epigenetic factors such as environmental condition and phytohormones are known to control sex determination in some other dioecious plant species such as *Mercia thomsoniana* (Louthan, 1981), *Centaurea solstitialis* (Gallagher, 1978) and *Artemisia triphylla* (Pokranc, 1981). Knowledge on the molecular and genetic control of sex determination has emerged only in the case of *Silene latifolia* with distinct Y chromosomal regions essential for the
suppression of pistil development and promotion of stamen developmental programme in male plants (Ye et al., 1991; Grant et al., 1994; Farooq et al., 1999).

In order to investigate the genetics of male sex determination and stamen development in dioecious plants, male-specific transcripts need to be isolated from developing flowers by cDNA subtraction. In *Solanum tuberosum* one of the DNAs identified, STAL1 had high DNA and amino acid sequence homology to the male sex-determining gene in *TASSEL-SHRED*. Both genes are expressed in male and not in female flowers, however, they do not share the same expression pattern (Lebel-Hambersack et al., 1997). Sex determination in cucumber (*Cucumis sativus*) is controlled largely by three genes, *Fm*, and *MK* and factors that induce ethylene biosynthesis, such as 1-amino-cyclopropane-1-carboxylate also enhance female sex expression (Troya et al., 1997). Sex determination in *Solanum tuberosum* seems to be controlled by heteromorphic sex chromosomes. The male determining Y chromosome overrides female development to suppress carpel formation and promote stamen development (Robertson et al., 1999).

The genome subtracted method "representational difference analysis" (RDA) has been used to identify male specific restriction fragments in the dioecious plant *Solanum tuberosum*. Male-specific restriction fragments are linked to the male sex chromosome Y-chromosomes. Four RDA derived male specific restriction fragments have been used to identify polymorphisms in a collection of mutant plants created by X-ray irradiation with either hermaphrodite or sexual flowers (Domais et al., 1996).

The dioecious white campion has been chosen as a working model for sexual development. In this species, sexual dimorphism has been achieved through two distinct developmental blocks, initiation of carpel development in male flowers, and early arrest of stamen differentiation in female flowers. The combined advantages of the dioecious system and the availability of a sexual mutant lacking both male and female reproductive organs have been exploited in a molecular subtraction approach using
male and asexual flower buds. This resulted in the cloning of 22 cDNA derived sequences expressed in stamens at distinct stages of development. 14 of these clones correspond to genes whose expression was detected in pre-metaphase stamens, a stage of development for which very little information is available. Further, the absence of similarities with database sequences for ten clones suggests that they represent novel genes. Functional analysis of each clone will enable their positioning within the reproductive organ developmental pathways. These clones are being exploited as developmental markers of early differentiation within the flower (Bernacou et al., 1997).

RAPID MARKER IN GENDER DETERMINATION

Sex determination is an important developmental event in the lifecycle of all sexually reproducing plants. Recent studies of sex determination in many plant species, from ferns to maize, have been fruitful in identifying the diversity of genetic and epigenetic factors that are involved in determining the sex of the flowers or individual. In those species amenable to genetic analysis, significant progress has been made towards identifying mutations that affect sex expression. By studying the interactions among these genes, pictures of how sex-determining signals were perceived to activate or repress male-or female-specific genes are emerging (Lauze and Banks, 1998).

For identifying sex-related genetic differences, male and female plant genomic DNA were analyzed individually. RAPD profile of the male plants generated by certain marker parents was distinctly different from the females by the presence of prominent male-plant associated bands. The RAPD technique (Welsh and McClelland, 1990; Williams et al., 1990) has been used to develop DNA markers linked to sexual phenotype in Petunia hybrida (Hermatz et al., 1994), Silene alba and Silene dioica (Taylor, 1994a and 1994b), Silene latifolia (Zhang et al., 1998), Silene vulgaris (Akstorm-Rapaport et al., 1998; Semenkov et al., 2003), Meconopsis integrifolia (Nayar et
et al., 1997; Krishnamoorthy et al., 1996; Azadirachta species (Gill et al., 1998), P. longum and Corokia cotonea (Macedo et al., 1999). Preliminary studies on the molecular basis of gerontic differentiation between the male and female P. longum plants, using randomly amplified polymorphic DNA technique has been investigated in this lab. Polymorphisms in the genome DNA was analysed and the presence of sex associated DNA markers in P. longum was reported (Banerjee et al., 1999).

Sex ratio in flowering and non-flowering plants from a single population of the dioecious species Solanum lycopersicum various accessions has been determined by Lynes et al., 1995: RAPD primer complementary to a DNA fragment on the male chromosome of S. lycopersicum was used to identify the sex of non-flowering plants. RAPD technique has been used in gender determination in Dioscorea elata (Yer-Ming Hu, 1995). Female sex-associated markers were developed in Fraxinus excelsior (Major Singh, 2002), sea-buckthom (Jarrosson et al., 1999) and in hemp (Terazini et al., 2001; Rode et al., 2005). Identification of hybrids in black pepper (Piper nigrum L.) was done using male parent-specific RAPD markers (George et al., 2005). In a few dioecious plants, morphologically distinct sex chromosomes (Ainsworth et al., 1998; Juarez and Banks, 1998), which are expected to play a crucial role in sex determination and differentiation have been observed. Several repeated sequences and a few genes have been isolated from sex chromosomes of two plant species, Solanum harrisii (Dorison et al., 1996; Matsunaga et al., 1996; Schetter et al., 1997; Gutman and Charlesworth, 1998) and Rhamnus xerophila (Tersis et al., 2000; Matsunaga and Kawano, 2002). However, the molecular function of sex chromosomes in sex determination and differentiation in plants still remains largely unclear.

Sex-linked genetic RAPD, AFLP or microsatellite markers have been discovered in the dioecious plant species Prunus vera (Hormaza et al., 1994), Hipsanthes lapidana (Polley et al., 1997; Dillenbecka spreta (Epolley et al., 1998), Hymenophyllum venosum (Peterson and Noltem, 1998), Hesperis officinalis (Chang and Sink, 1997; Seeleifer et al., 2000).
Carica papaya (Paraskev et al., 2000) and in Silene latifolia (Mulecay et al., 1992, Lyons et al., 1994, Zhang et al., 1998). In most cases, the genetic methods to identify gender have been developed for the purpose of plant breeding. However, genetic methods have been only rarely applied to investigate the sex ratios of natural plant populations (Lyons et al., 1995; Eppley et al., 1998) and apparently never before to studies involving seed populations as well (Kerptäinen, 2002).

CONVERSION OF RAPD MARKERS TO OTHER MARKERS

Sequence Characterized Amplified Region (SCAR) Markers

To increase reproducibility and ease of marker identification for routine analysis, it has been suggested that the RAPD markers should be converted to sequence characterized amplified region (SCAR) markers based on their DNA sequence, which could be detected through polymerase chain reaction (PCR) with longer sequence-specific primers (Paran and Michelmore, 1993).

Several of the sex-linked RAPD markers have been converted into SCAR markers i.e., in Asteriscus chorizasis (Gill et al., 1995), papaya (Oraskej et al., 2002), Salix torulosa (Gunter et al., 2001), and several resistance RAPD markers converted to SCAR (Paran and Michelmore, 1995, Arnedo-Andres et al., 2002). Male-specific SCAR markers developed on the basis of respective RAPD marker sequences in Homo sapiens (Polley et al., 1997), Asparagus officinalis, Aegilops claussena (Gill et al., 1998) and Cucumis sativus (Mandelbe et al., 1999); one in each species and five in Silene latifolia (Zhang et al., 1998; Gill et al. 1998) developed one female sex-specific SCAR marker in Asparagus by following a similar strategy.

Screening of a large number of RAPD primers has been necessary to score sex-linked markers in other dicot species. One female sex-linked RAPD marker (850 bp) has been identified in Parnassia nove (Kormazm et al., 1994). To identify RAPD markers
linked to the restorer gene used in the *Opuntia cedash* cytoplasmic male sterility of rapeseed, a total of 178 arbitrary 10-mer oligonucleotide primers have been screened in three pairs of bulk DNA. Four RAPD fragments that were completely linked to the restorer locus have been cloned and sequenced to develop SCAR ([Delourme et al., 1994]). BSA was used to determine RAPD markers in a specific interval in the middle of chromosome 6 of rice for tagging the photoperiod sensitivity gene. 80 primers were used to establish 14 markers tightly linked to the photoperiod sensitivity gene. RAPD markers were converted into sequence tagged sites by cloning and sequencing of polymorphic fragments and used for construction of physical maps ([Maune et al., 1995]). After screening 400 primers, a sex-associated SCAR marker in *Chara faucalis* ([Polley et al., 1997]) was identified. SCAR marker associated with a male-determining locus was developed after screening of 760 primers in *Asparagus* ([Wang and Shen, 1997]). In *Asplenium*, 10 male and 10 female plants were bulked by sex, using 158 decamer primers RAPD fragments were generated. A 2075 bp male-specific DNA fragment with OP06-14 primer was identified. Observations suggest that sex determination in *A. gossei* is genetic with no evidence of heteromorphic sex chromosomes ([Raju et al., 1998]). Bulked segregant analysis was used to identify RAPD markers in rapeseed from the fertile and sterile DNA bulks. Two polymorphic bands s1038-17231 and s1038-18240 were found linked to the specific locus. This was converted to SCAR markers, which facilitated selection on the restorer lines in rapeseed ([Wang et al., 2000]).

In *papaya*, sex diagnostic PCR assay for mass screening using SCAR markers by cloning and sequencing male specific RAPD fragment of 851 bp has been achieved ([Parawins et al., 2000, Georgopadysy et al., 2005]). Sex-specific DNA markers were developed in *A. ferox* tree mangostan ([Keshamounathy et al., 1996, Ganeshaiah et al., 2007]). In *papaya* a male and hermaphrodite specific RAPD marker developed with a 350 bp marker fragment for male and hermaphrodite plants. This has been converted into SCAR which is considered to be a suitable marker for the precise and rapid
diagnosis of sexes in papaya (González et al., 2002). Similarly RAPD products have been cloned, sequenced and converted to SCAR primers, and were used to correctly predict hermaphrodite papaya plants in a population of seedlings with an overall accuracy of 99.2% (Denny et al., 2002).

RAPD and SCAR markers were also developed for Coix neomexicanus (Arnedo-Andrés et al., 2002). Genetic markers linked to a female sex-determination locus in Solenostemon have been discovered through BSA using approximately 1,000 arbitrary primers. Two RAPD markers that were present in the common female progeny of these families were subsequently sequenced and converted into SCAR markers. The two SCAR markers are correlated with gender and are present in 96.4% of the female progeny and 2.2% of the males, providing evidence of linkage to a putative female-specific locus associated with gender determination in Solenostemon (Ganter et al., 2003).

DIFFERENTIAL DISPLAY and Non-Coding Region ESTs

In the absence of any genetic or sequence information of a closely related species or individuals showing morphological similarity and limited difference, differential display becomes an important technique to look into their genetic similarity and differences. Plants showing differences like disease resistance and susceptibility can be screened by this technique and a sequence homology search is subsequently carried out. Differential display, since its introduction in 1992, has found wide applications in almost all areas of research. It is a fingerprinting technology that facilitates the identification of mRNAs in a cell or tissue and can be used to compare mRNA expressions in many samples (Liang and Pardee, 1992 and 1997; Liang et al., 1992 and 1994; Jackson and Mark, 1997; Zhang et al., 1997; Scott et al., 2002). Thousands of genes embedded in the genome of an organism are selectively expressed into the mRNA and proteins that gives rise to different tissues or organs. Any abnormality in this causes a variety of pathological alterations or diseases. STS obtained by differential
display is a short unique fragment of DNA (1-300 bp) and the polymorphic STS markers are commonly used for genomic analysis of plants.

The isolation and characterization of differentially expressed genes becomes the first step in understanding the unique properties or abnormalities. One of the better ways to achieve this is by reverse transcription of mRNA with anchored primers followed by amplification of cDNA using arbitrary primers. The products are then run in a sequencing gel for high resolution of the amplified cDNA. This usually results in tagging a few bases which is sufficiently unique to identify mRNA and getting it separated from others by size. These differently displayed fragments can be screened and sequenced to develop specific primers which can be used to screen plants. Male specific genes from dioecious white campion were identified by differential display (Welsh et al., 1992; Soini et al., 2002).

Expressed sequence tags (ESTs) are subsets of STS derived from cDNA clones. ESTs can serve the same purpose as the random STSs with an advantage that ESTs are derived from expressed genes that are from spliced mRNA, which is usually free of introns, as well as repetitive DNA. ESTs have the advantage of representing real functional genes and are therefore more useful as genetic markers than anonymous non-functional sequences. In species having large genomes, cDNA sequences and ESTs are advantageous for comparative genomic analysis and in gathering information on genomic structure.

PROTEOME ANALYSIS BY MALDI-TOF

Genetic diversity studies have been widely used for varietal identification, breeding programmes, and cultivation. Assessments of genetic similarity of various crops and species have been performed using morphological data, quantitative genetics DNA studies (Rodriguez et al., 1999) and proteome analysis (Pesch et al., 1994, Pesch et al.,
In this genomics era, researchers are turning to methods of analysis to determine protein functional information on a large scale with high quality results. Functional proteomics has evolved as a need to understand and investigate expressed proteins of an organism (Washburn and Yates, 2000). Protein analysis and characterization has been done by mass spectrometry, which is further applied to protein structure studies to understand functional proteomics (Teoh, 2000; Gygi et al., 2000) and DNA sequencing (Koster et al., 1996).

Mass spectrometry offers advantages over gel electrophoresis separation and various forms of hybridization, particularly for automated rapid and large-scale DNA and RNA screening. The use of electrophoretic mobility of fluorescence of a label is only indirect measure, but mass is an intrinsic property of molecule, as a result mass spectrometric assay are intrinsically more accurate. The nuclear genome contains most of the genetic information of a plant in a set of chromosomes, each composed of a single DNA molecule. Genomes of different organisms vary in terms of total DNA content (genomic size), ploidy level, chromosome number, total recombination distance, nature and number of functional genes. At the molecular level, chromosomes are composed of DNA and proteins. The DNA carries the genetic information, while the protein compounds provide enzymatic and structural function, important to the replication, recombination and segregation of the chromosome. The main part of the chromosome contains a mixture of coding sequences (exons) intragenic and intergenic non-coding sequences (introns, regulatory sequences, tandem repeats (mainly minisatellites, microsatellites and VNTRs) and other dispersed repeats. Tandem repeats have been used in genomics research and have shown a great potential in genome mapping (Filip et al., 2004).
TISSUE CULTURE OF *PIPER LONGUM*

In the recent past, industrial demand for *P. longum* is continuously increasing which is largely met by collection from its natural habitat which leads to endangering the genetic variability and natural resource of this species. As these plants are excessively utilized from its natural habitat, the species has now become very rare in Kerala and endangered in Tamil Nadu (Nair, 2000; Raveendran and Ved, 2000). Attempt at tissue culture has been done in *P. nigrum* (Misra et al., 2004) and to a lesser extent in *P. longum*. This includes reports on regeneration (Samasar and Nair, 1991; Samaran et al., 1995; Bhat et al., 1995; Philip et al., 2000; Seniya and Das, 2002). Identification of plants with superior therapeutic and agronomic traits and development of a rapid method for clonal multiplications of different genotypes are extremely necessary for the promotion and cultivation of this spice as an under-shrub in tropical plantations. Long pepper from Assam (*Assam variety*) is known for its excellent therapeutic properties. Kerala Agricultural University has released a variety, named ‘Rosam’, which bears bold female spikes (Philip et al., 2000).

Long pepper plants are normally propagated by vegetative means, since conventional propagation is beset with problems of poor seed viability, low percentage of germination and delayed rooting of vegetative cuttings. The development of an easy and standard protocol for clonal multiplication could very well substantiate the cultivation efforts through increased availability of planting material of superior varieties. However, micropropagation technique has not been demonstrated on diverse genotypes of long pepper. A large number of plants are produced through tissue culture for which excised shoot tips, leaves and stem pieces have been used as explants. The establishment of protocols for in vitro propagation of long pepper through shoot multiplication and direct regeneration offers a potential system for improvement, conservation and mass propagation of this important medicinal plant. For the
development of a standard and efficient method of plant regeneration, node, internode and leaf explants have been used (Philip et al., 2000).

Tissue culture on long pepper has been reported for culture of shoot tips, leaves and stem explants (Sorensen and Pas, 2002). MS basal medium supplemented with BA, Kt, IAA, NAA and Thidiazuron individually or in combinations induced regeneration of plants with and without callus formation. Multiple shoots have been induced directly from leaf explants of long pepper using combinations of BA, Kt and coconut water in MS medium (Sarasa and Nair, 1991; Sarasa et al., 1992). Bud formation at the base of leaf explants has been described earlier by many, in other plants (Hosokai and Asakura, 1980; Cramer et al., 1990). It is possible that new shoots originating from axillary meristems have higher potential for bud formation (Hosokai and Asakura, 1980; Kukulczanka and Czeczek, 1989). Earlier reports on plant regeneration in long pepper (Sambrose et al., 1989) described maximum regeneration from root explants. RAPD markers have been used for assessing genetic diversity among in vitro propagated plants towards conservation of long pepper (Prami et al., 1995).

PURPOSE AND OBJECTIVES OF THE PRESENT STUDY

The Indian medicinal plants-based industry is growing at the rate of 7-15% annually. The value of medicinal plants-related trade in India is estimated at Rs. 5000 crores (US dollars) (Bhaduri and Verma, 2000; Joshi et al., 2004). Global trends leading to increased demands of medicinal plants for pharmaceuticals, phytochemicals, nutraceuticals, cosmetics and other products is an opportunity sector for Indian trade and commerce. Herbal drug technology involves all the steps that are involved in converting botanical materials into medicines, where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge remains important. Correct identification and quality assurance of the starting material is, therefore, an
essential prerequisite to ensure reproducible quality of herbal medicine that contributes to its safety and efficacy (Joshi et al., 2004).

The monoecious and dioecious plants manifest the difficulty in distinguishing the factors that influence sex determination from the factors that mediate the resulting floral differentiation programs. Plant growth regulators such as auxins, cytokinins and GAs influence sex differentiation but have not been demonstrated to be involved in decisive stages during normal development. In both types of system, maleness and femaleness appear to be achieved through the suppression of the differentiation programme for the opposite sex. This suggests that sex determination acts through genes regulating the relative activities of the male and female differentiation pathways, possibly using known growth regulators as messengers (Joshi and Nelson, 1999). In many dioecious species, their sexes remain indistinguishable until the flowering stage and maintain their sexual identity throughout. Among few dioecious plant species, female plants are commercially and medicinally important. Hence, knowing their sexual identity in the seedling or vegetative stages is advantageous.

Over the past few years, molecular genetic markers have been developed into useful tools for studies of genetic diversity and genetic mapping. These methods of detection include restriction fragment length polymorphisms (Bolstein et al., 1980) and the more recent technique based on the polymerase chain reaction (Ledbetter et al., 1990). Randomly amplified polymorphic DNA combines PCR and random primers to amplify genome DNA and produce a fingerprint. Among dioecious plants including *Piper longum* it is difficult to segregate female and male populations during seedling or vegetative stage and hence the present study focuses on the following objectives. Tissue culture and molecular studies have been simultaneously carried out to compare and contrast cultivated and tissue-cultured plants of long pepper for understanding the fidelity of sex markers.
OBJECTIVES:

- To assess genetic variation and diversity of *Piper longum*.
- To investigate the molecular bases for dioecious nature of plants.
- To find out RAPD markers for sex identification at the seedling-vegetative stage.
- To convert RAPD markers into SCAR markers. (SCAR markers are sequence-specific, are not biased to minor variables in experimental conditions, are reproducible, reliable, simple to use and suitable for multiplex PCR analysis).
- Screening of plants including the tissue cultured ones using the above markers to determine male and female populations and
- To regenerate *Piper longum* through clonal propagation and analyze the efficiency of molecular markers to differentiate sexes.