CHAPTER III
GENETIC DIVERSITY

3.1. Introduction
3.1.1. Genetic diversity

Genetic diversity is widely recognized as the key component for long term survival of the species. It is the foundation of sustainability because it provides raw material for adaptation, evolution and survival of species and individuals, especially under changed environmental and disease conditions (Erikson et al., 1993). Estimation of genetic diversity of plant population has been recognized as elementary topic not only to delineate in situ and ex situ conservation strategies (Holsinger and Gottlieb, 1991), but also to establish forms of rational and sustainable exploitation of genetic resources (Chalmers et al., 1992).

The ability of a particular genotype to tolerate drought or inundation, grow in poor or rich soil, to act against insects, pest or disease, to offer higher protein yield or to produce a better tasting food are traits passed on naturally by its gene. The genetic substances constitute the raw material that plant breeders and biotechnologists utilize to produce new varieties or cultivars. Without this diversity, the ability of better adaptation to over changing conditions and needs will be lost (Menini, 1998). Information on genetic diversity and relationships within and among crop species and their wild relatives is essential for the efficient utilization of plant genetic resource collections (Chan and Sun, 1997).

An assessment of the nature and extant of intraspecific genetic diversity and a deeper understanding of systematics have diverse applications in utilization and conservation of genetic resources and taxonomic studies (Kavitha et al., 2010). Analysis of genetic relationships in crop species is an important component of crop improvement programs, as it serves to provide information about genetic diversity, and is a platform for stratified sampling of breeding populations. Investigation of genetic diversity of germplasm collections can facilitate reliable classification of accessions and identification of subsets of core accessions with possible utility for specific breeding purpose (Mohammad and Prasanna, 2003).
3.1.2. DNA based Molecular Markers

Genetic information that makes up genes in higher plants is stored in the DNA molecules. There is such an enormous amount of DNA in higher plant cell, that no two organisms are likely to be identical in the DNA base sequence. Natural variation in DNA sequence can be detected in several ways. Advances in molecular biology during the last two decades have provided a new class of genetic markers that can be used for plant classification and breeding. Polygenic characters are very difficult to analyze using molecular markers. Molecular markers can be used for characterization of germplasm, variation of genetic relationship, polygenetic and evolutionary studies and marker assisted selection (Waycott and Fort, 1994; Gilbert et al., 1999; Smith and Bevies, 1998). DNA based marker will be more accurate in determination of relationship between accessions that are too close to be accurately differentiated by others (Hu and Quires, 1991; Chalmers et al., 1992; Demeke et al., 1992).

3.1.3. The polymerase chain reaction (PCR)

The PCR is a powerful technology developed by Mullis and Faloona (1987) who demonstrated that oligonucleotide primers could be used to rapidly amplify specific segments of DNA. Rightly known as molecular photocopying, PCR exploits the remarkable property of natural polymerase enzymes to copy the genetic material, which can be either DNA or RNA. The technique is useful in detecting polymorphism but the PCR based polymorphism assay known as Amplitude Sequence Polymorphism (ASP) requires target DNA sequence information for the design of amplification primers (Skolnick and Wallace, 1988). The time and cost of obtaining this sequence information is prohibitive for many large scale genetic mapping applications.

3.1.4. Random amplified polymorphic DNA (RAPD)

Williams et al. (1990) developed a new DNA polymorphism assay based on the amplification of random DNA sequence with single primers of arbitrary nucleotide sequence. The primers detected polymorphism in the absence of specific sequence information and the polymorphism, which functioned as genetic marker was called RAPD marker. Their result suggested that the minimum useful primer length was an oligonucleotide of nine bases and a GC content of 40 percent or greater was required to generate detectable levels of amplification products. They also reported that single base changes in the arbitrary primer could cause a complete change in the set of
amplified DNA segments. The amplification of DNA using arbitrary 10-base oligonucleotide primers has been described as a strategy to detect RAPD in many eukaryotic organisms (Williams et al., 1990). Polymorphism generated by random amplified polymorphic DNA analysis has been used for fingerprinting (Cannolly et al., 1994) and evaluating genetic relationship among cultivars (Stiles et al., 1993).

The information obtained using RAPD is extensively used for identification of germplasm, screening of duplicate, assessing genetic diversity and monitoring the genetic stability of conserved germplasm (Rajput et al., 2007). The usefulness of RAPD technique for detecting genetic variation among cultivars and identifying germplasm is well established (Welsh and Mc Clelland, 1990). RAPD markers are based on the amplification of unknown DNA sequences using single, short, random oligonucleotide primers, therefore RAPD Polymorphism is the reflection of variation of the whole genomic DNA and would be a better parameter to measure the pattern of genetic diversity of the rare and endangered plants (Jayram and Prasad, 2008). It is simple, cost effective and a powerful tool in the analysis of plant genome characterization. Although, RAPD is criticized for its low reproducibility (Hansen et al., 1998; Virk et al., 2000) but it is overcome by optimization of the RAPD reaction and maintenance of stringent conditions. RAPD has, therefore been extensively used in assessing genetic diversity and relationship measures in various plant species (Bauvet et al., 2004; Upadhyay et al., 2004).

RAPD method for generating DNA fingerprints elucidates genetic differences at the DNA sequence level which result from evolutionary mechanism such as DNA deletions, additions, substitution, repetitions and translocations. RAPD is very useful in identifying and estimating the genetic diversity among closely related cultivars and wild species. They contribute much for establishing taxonomic discrimination and phylogenetic relationship among taxa. The initial evaluation of genetic variation in the species among taxa, among populations and among zones, among populations and among individuals can be effected by RAPD markers. RAPD markers were used and the level of polymorphism revealed the immense potential of RAPD in the genetic fingerprinting of Pigeonpea (Rataparkhe et al; 1995). Phylogenetic relationship of Ocimum (Singh et al., 2004) and Salvia (Khalil et al., 2005) etc. have been determined by analyzing their genetic pattern using RAPD.
RAPD - PCR has entered plant research in a revolutionary way due to its technical simplicity and genetic informativeness. This molecular marker using PCR in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals (Xena de Enrech, 2000). The quality of genomic DNA is a major factor that affects the reproducibility of RAPD patterns by affecting the primer annealing. Micheli et al. (1994) reported that ethanol precipitable contaminants like low molecular weight DNA and RNA in the genomic DNA preparation after the formation of productive template primer complexes and hence influence the reproducibility of RAPD patterns.

Stift et al. (2003) did the comparative study of RAPD fragments separation in agarose and polyacrylamide gels. They found that better resolution of the bands and more number of polymorphic bands are obtained in polyacrylamide gels. It has been reported that RAPD markers differ according to variations in experimental conditions. Wolf et al. (1993) reported that RAPD markers vary according the changes in concentrations of MgCl₂ and the type of thermal cycler, while Schiewater and Ender (1993) reported that the variation occurs with change in quantity and quality of Taq polymerase enzyme.

3.1.5. Geographical background of collection site

The geographical position of Kerala has its own uniqueness and its landscape its own beauty. Kerala is a land that lies between the high hill ranges of Sahyadris in the east and the Arabian sea (Lakshadweep Sea) in the west. Kerala, gifted with mountains, valleys, trees, a wide variety of plants and grasslands has a share of just 1.2% of the total area of India. This region, with a varying topography, fertile soil and an ideal climate has been the abode of man from time immemorial.

Kerala is located between latitudes 8°.17’. 30”’ N and 12°.47’. 40”’ N and longitudes 74° .27’. 47” E and 77° .37’. 12” E. It has an area of 38,863 sq. km. Climate of Kerala is different from that of the other Indian States. According to meteorological data, Kerala receives rain around 286 days in a year. Kerala’s average annual rainfall is about 300 cm. South West monsoon (June to September) and North East monsoon (October to December) are the two rainy seasons of Kerala. Kerala’s average maximum daily temperature is about 36.7°C, the minimum is 19.8°C. Red
soil, laterite soil, coastal alluvium, riverine alluvium, forest soil and black soil are the type of soils in Kerala. Laterite soil covers an area of about 68% of the total area of the state. [http://www.prokerala.com/kerala/geography.htm]

Eastern Kerala consists of land encroached upon by the Western Ghats; the region thus includes high mountains, gorges, and deep-cut valleys. The wilded lands are covered with dense forests, while other regions lie under tea and coffee plantations or other forms of cultivation. Here, the Western Ghats forms a wall of mountains penetrated near Palakkad; here, a natural mountain pass known as the Palakkad Gap breaks through to access inner India. The Western Ghats rises on average to 1500 m elevation above sea level. Just west of the mountains lie the midland plains, comprising a swathe of land running along central Kerala. Here, rolling hills and shallow valleys fill a gentler landscape than the highlands. Kerala’s coastal belt is relatively flat, teeming with paddy fields, groves of coconuts trees, and heavily crisscrossed by network of inter-connected canals and rivers.

Kerala is divided into three geographical regions: - highlands, midlands and lowlands. The highlands slop down from the Western Ghats (also known as Sahyadri) which rise to an average height of 900 m, with a number of peaks well over 1800 m in height. It is 18650 sq.km in area and account for 48 percent of the total land area of Kerala. The midlands, lying between the mountains and lowlands, are made up of undulating hills and valleys. It is 16200 sq. km in area i.e. about 40 percent of the total land area. Lowlands are also known as the coastal area. It covers an area of almost 4000 sq.km. It is made up of numerous shallow lagoons known locally as kayels rivers deltas, back water and shores of the Arabian sea, and essentially a land of coconuts and rice. [http://www.maps of India.com/maps/Kerala/geography-and history].

The fascinating land of Kerala comprises of 14 districts, which figure as the leading revenue earners of India. Taking into account geographical, historical and cultural similarities the districts are generally grouped as North Kerala (Kasarakod, Kannur, Wayanad, Kozhikode and Malappuram), Central Kerala (Ernakulam, Palakkad, Thrissur and Idukky) and South Kerala (Trivandrum, Kollam, Alappuzha, Pathanamthitta and Kottayam) [http://www.maps of India.com/maps/Kerala/geography-and history].
3.1.6. Scope of the study

The information on genetic diversity and relationship within and among crop species is essential for the efficient utilization of plant genetic resource collection (Irwin et al., 1998). For the efficient conservation and management the genetic composition of the species in different geographic locations need to be assessed (Jayaram and Prasad, 2008). DNA based markers provide new tool for ecological and genetic studies of evolutionary processes (Hasen et al., 1998). Newer markers such as microsatellites and RAPD provide more detailed genetic information due to either the increased genetic validity of loci or the greater number of the available loci (Cruzan, 1998). These markers have successfully been used to estimate levels of relatedness among the individuals (Dow and Ashely 1996). Due to technical simplicity and speed, RAPD methodology has been used for diversity analysis in many red listed plants (Li al., 2002; Fu et al., 2003a).

The wide geographical and climatic distribution is indicative of the fact that there exists a tremendous genetic diversity in Kaempferia galanga which needs to be indentified and catalogued. The evaluation of K. galanga genetic diversity and relationship is still insufficiently carried out. As the genus has high ecological, economical and medicinal value, an accurate assessment of genetic diversity and relatedness will be helpful for efficient management of this genus. Therefore this study is concerned with the evaluation of the genetic diversity and relationship of K. galanga accessions by RAPD analysis.

3.2. REVIEW OF LITERATURE

3.2.1. RAPD for genetic diversity analysis

Chan and Sun (1997) examined the genetic diversity and relationship of 23 cultivated and wild Amaranthus species. More than 100 RAPD fragments were generated with 27 arbitrary 10 – base primers. On average, 39.9% of the RAPD fragments were polymorphic among accessions within each crop species. Lerceteau et al. (1997) evaluated the extent of genetic variability among Theobroma cacao accessions. Gheardi et al. (1998) analysed the genetic diversity by screening DNA from individual plants of eight cultivated and natural populations of Medicago sativa and M. falcata using the RAPD method. Five primers were used and 64 intense bands
were scored. Most of the loci were seen to be highly polymorphic whereas very few populations with specific polymorphism were identified.

*Plantago major* from several Scottish and Dutch locations were surveyed for their genetic variation using PCR markers namely RAPD analysis (Wolf and Morgan, 1998). Genetic variation among 43 date palm (*Phoenix dactylifera* L.) accessions including 37 accessions from Morocco and 6 cultivars from Iraq and Tunisia was studied using Random Amplified Polymorphic DNA markers (Hasan *et al.*., 1998). All 43 analysed genotypes were distinguishable by their band patterns. RAPD based genetic distance was used to determine the relationship between accessions.

Gallois *et al.* (1998) have developed a RAPD protocol for members of the *Fagaceae* family and have studied RAPD variation among several polulations of *Fagus sylvatica*. Genetic variation in cucumber (*Cucumis sativus* L.) was assessed by Random Amplified Polymorphic DNA (Thomas and Jack, 1999). One hundred and eighteen *C. sativus* accessions were analysed using variation at 71 RAPD loci. Each accession had a unique marker profile indicating that RAPD analysis was useful in genotypic differentiation.

Boehm *et al.* (1999) estimated genetic difference among eleven cultivated and eight wild type populations of North American Ginseng and four cultivated populations of South Korean Ginseng using RAPD markers. Evaluation of germplasm with 10 decamer primers resulted in 100 polymorphic bands. Pradeepkumar *et al.* (2001) did the molecular characterization of *Piper nigrum* L. cultivars using RAPD markers. Thirteen land races and 9 advanced cultivars were characterised using 24 selected primers which generated 372 amplification products. Cultivar specific bands could be obtained for most of the cultivars and varieties studied. Sangwan and Sangwan (2001) reported the randomly primed PCR based assessment of genetic variability in a population of *Artemisia annua*, an antimalarial medicinal plant. They also analysed the association of polymorphic markers with economic characters like essential oil content and concentration of artemisinin.

Qian *et al.* (2001) investigated genetic variation within and between five populations of *Oryza granulata* from two regions of China using RAPD and ISSR markers. Twenty RAPD primers were used and amplified 199 reproducible bands with
61 polymorphic groups. Gang et al. (2002) reported the use of RAPD marker analysis for identification of polymorphic markers for bacterial wilt resistance between resistant and susceptible bulk DNA of Solanum phureja using 300 random primers. The primer OPG 09 gave a 960 bp reproducible band in resistant clones in the population.

Pawankumar et al. (2003) carried out a genetic diversity study in a set of 30 elite cotton germplasm lines using RAPD markers and morphological characteristics. Fu et al. (2003b) employed RAPD markers to analyse the genetic variation in 54 North American flax cultivars. A total of 84 polymorphic bands could be generated using 16 arbitrary primers. The results indicated that the overall RAPD variation present in the flax cultivars was relatively moderate.

Shashidharan et al. (2003) identified RAPD analysis as an efficient marker technology for estimating genetic diversity and relatedness in sandalwood. They screened 51 genotypes of Santalum album procured from different geographical regions in India and three exotic lines of S. spicatum from Australia using 11 selected Operon primers. Rare and genotype specific bands could be identified and cluster analysis separated the Indian genotypes from the Australian and also indicated that the sandalwood germplasm within India constituted a broad genetic base.

Random Amplified Polymorphic DNA analysis was carried out in 29 Indian mango cultivars comprising popular land races and some advanced cultivars at the NRC for DNA finger printing (Karihaloo et al., 2003). The PCR amplification with 24 primers generated 314 bands and Jaccard’s similarity between pairs of cultivars ranged between 0.318 and 0.75. In the dendrogram majority of the cultivars from North and Eastern regions of India clustered together and separated from the southern and western cultivars.

RAPD profiling of 33 collections of Phyllanthus amarus from various parts of India was carried out by Jain et al. (2003). Analysis through UPGMA revealed 65 parent variations among the accessions. ISSR and RAPD markers were used to analyse genetic distance among Hordeum vulgare populations from west Turkey by Bahattin (2003). A total of 55 polymorphic loci were found using 65 primers. Two distinct cluster groups were clearly established among populations.
Chandrashekara et al. (2003) reported the use of RAPD marker analysis to determine the extent of interspecific genetic diversity in tomato. RAPD assay was carried out using 12 random decamer primers and four accessions representing four species of tomato namely *Lycopersicon esculentum*, *L. pimpinellifolium*, *L. glandulosum* and *L. hirsutum*. Sharma et al. (2004b) reported the molecular analysis of variability in *Podophyllum hexandrum*, an endangered medicinal herb. They characterized 30 plants collected from different areas in Himachal Pradesh using RAPD markers. Out of the 40 random primers tested, 7 produced amplification giving a total of 76 RAPD markers.

Genetic diversity in traditional Sali rice germplasm of Assam was analysed through RAPD markers. 51 rice accessions were characterised based on 72 RAPD markers. The Jaccard’s similarity coefficient was found to be 0.515 indicating a high level of diversity (Barooah and Sarma, 2004). Upadhyay et al. (2004) analysed genetic diversity and genetic relationship among 20 Indian coconut accessions. The 8 primers yielded 77 markers with an average of 9.6 markers per primer. The within accession genetic diversity ranged from 0.057 to 0.196.

Das et al. (2004a) reported the use of RAPD technique to evaluate the genetic diversity among 12 cultivars and root stocks of *Citrus* in North East India. Ten selected decamer primers produced 97 amplified fragments, all of them except one being polymorphic and 11 were unique to some germplasms. The genetic diversity was found to be low to moderate and cluster analysis classified the 12 germplasms into two major clusters.

Uma et al. (2004) studied the genetic diversity and phylogenetic relationship among indigenous and exotic silk group of bananas using RAPD markers. Thirty five DNA fragments were amplified from 25 silk group representations using four random primers. The average polymorphism among the amplified products was 51.2 % thus indicating a considerable variation at the DNA level. In another study Onguso et al. (2004) applied RAPD to estimate genetic relationship among 20 selected banana cultivars from different regions of Kenya. Analysis using 19 random primers placed all the 20 cultivars into one cluster showing that they are related.
Das et al. (2004b) did the RAPD profiling of 25 elite clones of mandarin orange selected from seven locations in three states of North Eastern Himalayan regions of India. Using ward’s cluster analysis, the 25 plants were classified into two major clusters and further into sub-clusters. The study confirmed the existence of wide genetic diversity in mandarin orange. Dongre and Kharbikar (2004) reported the RAPD finger printing of 25 cotton accessions from Africa, Australia, the USA and India using 86 arbitrary primers. The RAPD analysis using SIMQUAL – Dice coefficient of NTSYS PC showed that the 25 accessions could be split into two groups of 24 and one accession at 67 % similarity. The first group was further split into sub-clusters.

The DNA fingerprinting in Hydrastis canadensis, an endangered perennial wild flower native to North America was performed using RAPD analysis. Samples collected from different areas including cultivated and wild populations were studied. The cultivated material showed 72 – 86 % similarity while the wild populations have 20 – 67 % similarity (Kelley et al., 2004). Singh et al. (2004) examined the genetic relationship among 30 germplasm accessions of Ocimum belonging to 5 different species using RAPD markers. A high degree of polymorphism up to 98.2% was observed and the UPGMA cluster analysis grouped all the accessions into two major clusters corresponding to previously report botanical groups.

Reis and Grattapaglia (2004) studied the RAPD variation in a germplasm collection of Armeria, an endangered tropical tree. Genetic similarity in the germplasm consisting of 9 collections from different geographical areas was estimated with 83 RAPD markers. The Principal Coordinated Analysis (PCA) showed that there was no definite clustering among individuals from the same collection area. Suma and Balasundaran (2004) reported genetic diversity of 8 Santalum album L. provenances of India based on RAPD analysis.

A study was undertaken with RAPD markers for genetic diversity estimation in 59 cotton cultivars belonging to 4 cultivated species of cotton (Rana and Bhat, 2005). The selected 18 RAPD primers produced a total of 25 % amplicons which generated 97.21% polymorphism. Justin et al. (2005) estimated intra-population genetic variation and relationship among different Tylophora indica populations of ten
accessions from Tirunelveli and Kanyakumari district of Tamil Nadu using RAPD markers. The analysis of molecular variance revealed that considerable level of variations is observed in the species.

Keshavachandran et al. (2005) reported the genetic fingerprinting of *Piper nigrum* and *P. longum* cultivars using RAPD marker. Fourteen landraces and three advanced cultivars of *P. nigrum* and 11 land races and one advanced cultivar of *P. longum* were amplified using 10 sets of random primers to give 119 amplification products. The analysis indicated that the accessions could be differentiated based on their RAPD profiles. Nazeem et al. (2005) used RAPD and AFLP techniques to assess the genetic variability in 49 black pepper varieties. They observed an average similarity of 63% among the accessions.

Dey et al. (2005) have reported the genetic diversity analysis of aromatic rice suing RAPD markers. 38 aromatic rice lines and 2 non–aromatic controls were screened using 5 random primers giving 44 amplification. Products of which 41 were polymorphic. Results indicated that there was considerable amount of genetic diversity within the genotypes assessed and the 5 RAPD primer generated polymorphism clearly indentified each of the 40 rice genotypes distinctively.

Assessment of genetic variability in *Indigofera linnaei* L. was carried out by John De Britto and Nirmal Kumar (2006). The five primers used to analyse genetic variability in *I. linnaei* resulted in a total of 35 polymorphic bands. The genetic distance between the population ranged from 0.2132 to 0.9876 and genetic identity ranged from 0.3421 to 0.6765.

The distribution of genetic variation as revealed by Randomly Amplified Polymorphic DNA (RAPD) markers was examined in *Oldenlandia umbellata* in Tamil Nadu, India (John De Britto et al., 2006). It was observed that these medicinal plants possess a considerable amount of genetic variation. The 5 primers used to analyze genetic in *O. umbellata* resulted in a total of 53 polymorphic bands (loci). The genetic distance between the populations ranged from 0.2093 to 0.6381 and the genetic identity ranged from 0.5283 to 0.8113.

The introgressed cultures of *Gossypium hirsutum* were evaluated for their genetic diversity using molecular markers that could discriminate the genetic
relatedness and pedigree through RAPD analysis (Saravanan et al., 2006). Highly polymorphic markers could be identified through primers OPAL 20, OPAK 20 and OPAM 07. Assessment of genetic diversity of Lycoris longituba (Amaryllidaceae) was detected by RAPDs (Chuan et al., 2006). For the 180 individuals of L. longituba from three populations, 12 selected RAPD primers produced 94 reproducible and clear amplification bands of which 62 (65.96 %) were polymorphic which indicated that L. longituba had a high level of genetic diversity.

Molecular marker based genetic diversity analysis of Curcuma species from India was carried out by Syamkumar and Sasikumar (2007). Thirty nine RAPD primers yielded 376 bands of which 352 were polymorphic. Kumar et al. (2007) evaluated the genetic diversity in Cymbopogon species using PCR based functional markers. Costus speciosus were collected from different places in the Bay Islands and profiled involving RAPD markers to assess genetic diversity at genomic level (Asit et al., 2007). Genetic variability among 24 rice genotypes from Assam was assessed employing random amplified polymorphic DNA (Ninon et al., 2007). A total of 81 RAPD markers were generated with 92.5% polymorphism.

Chandrashekara et al. (2007) carried out genetic diversity analysis of elite pearl millet inbred lines using RAPD and SSR markers. The 20 random primers generated 127 amplicons of which 120 were polymorphic at an average of 6.00 polymorphic amplicons per primer. Iqbal et al. (2007) estimated the degree of genetic divergence in 7 wheat genotypes, 6 exotic genotypes and one local variety through random amplified polymorphic DNA methodology. A total of 112 DNA fragments were generated by the 15 random primer with an average of about 7.4 bands per primer. Among the 112, 50 fragments showed polymorphism among the 7 wheat genotypes.

ISSR and RAPD marker assessed genetic variation of Aerides maculosum – an epiphytic orchid from Goa, India were conducted by Parab et al. (2008). Among the 13 primers tested for molecular analysis of A. maculosum 100% polymorphic pattern was obtained with 7 RAPD primers. Kiran et al. (2008) used the RAPD approach to assess the quantum of genetic variation among the accessions of Hypericum perforatum at genomic level. Random Amplified Polymorphic DNA markers were used to evaluate the genetic diversity in 70 cowpea accessions collected throughout
Benin (Zannou et al., 2008). The genetic diversity among the cowpea cultivars investigated was large and the RAPD proved to be a useful technique to characterize it. Based on the molecular variance the fixation index suggests a large differentiation of cowpea cultivars in Benin. Moyib et al. (2008) used Random amplified polymorphic DNA primers to assess genetic diversity in twenty four accessions of Nigerian collection of African Yam bean (Sphenostylis stenocarpa).

Martin et al. (2008) carried out genetic diversity study of endangered Betula pendula populations from different geographical locations using RAPD markers. Esmail et al. (2008) carried out genetic diversity in elite cotton germplasm. 21 cotton genotypes were subjected to RAPD analysis. 23 primers showed good amplification. A total of 113 scorable bands were detected among which 96 bands (84.95%) were polymorphic. Roopa et al. (2008) used RAPD profiles to identify the extent of diversity among 54 accessions of mung bean that included both improved and local races. Out of the 40 primers screened, 7 primers generated 174 amplification products with an average of 24.85 bands per primer. The RAPD profiles were analysed for Jaccard’s similarity coefficient that was found to be in the range from 0 to 0.48 indicating the presence of wide range of genetic diversity at molecular level.

Genetic variation in ten indigenous populations of Medicago sativa from Azerbaijan was investigated by randomly amplified polymorphic DNA (RAPD) analysis (Shahin et al., 2008). A total of 80 and 78 fragments were scored using ten arbitrary primers for individual and bulk analysis respectively. The percentage of polymorphic loci was 67.95% for bulk analysis whereas for individual based analysis it was 100%.

The utility of RAPD markers in assessing genetic diversity and phenetic relationships in Persea bombycina, a major tree species for golden silk (muga) production, was investigated using 48 genotypes from northeast India (Brijmohan, 2009). Thirteen RAPD primer combinations generated 93 bands. On average, seven RAPD fragments were amplified per reaction. In a UPGMA phenetic dendrogram based on Jaccard's coefficient, the P. bombycina accessions showed a high level of genetic variation, as indicated by genetic similarity.
The random amplified polymorphic DNA (RAPD) markers were used to assess genetic diversity in *Sesamum indicum*. RAPD technique was carried out in a set of 10 sesame germplasm collected from different regions of Sudan. A total of 64 polymorphisms (6.4 polymorphic markers per primer) out of 75 reproducible products (7.5 fragments per primer) were obtained from the 10 primers used. The number of bands per primer ranged from 4 to 13, whereas the number of polymorphic bands ranged from 3 to 12, corresponding to 66.6% of the amplification products. Low level of genetic similarity was observed in the collected accessions. Unique bands were observed with the 10 primers. UPGMA clustering resulted in two major groups (Abdellatief et al., 2008).

Zhang *et al.* (2008) analysed the genetic variation in *Erianthus arundinaceum* by random amplified polymorphic DNA markers. One hundred and twenty seven bands were detected, of which 89 were polymorphic (70.07%). Accession relationship were estimated through cluster analysis (UPGMA) based on RAPD data. Zannou *et al.* (2009) analysed the genetic variability in Yam cultivars from the Guinea Sudan zone of Benin assessed by random amplified polymorphic DNA. The amplified bands revealed high polymorphism. The study showed that the genetic diversity changed along a spatial gradient.

RAPD markers were employed to assess genetic diversity in ten maize cultivars, *viz.* 4 hybrids and 6 composites (Poonam *et al.*, 2008). Fourteen out of the fifteen random primers screened revealed polymorphism among the genotypes. Most of the primers revealed single polymorphic band and 92.92% of the products were polymorphic. Seventy four scorable fragments were obtained with an average of 5.3 bands per primer and the average number of polymorphic bands found to be 5.1. Based on polymorphism 5 RAPD primers OPD-05, OPC-08, OPP-16, OPE-03 and OPF-17 were found to be highly discriminative. Genetic similarity based on jaccard's similarity coefficient ranged from 0.214 - 0.725, indicating narrow genetic variability among the genotypes based on RAPD markers. The ten cultivars of maize formed two major clusters in the dendrogram. The studies comprising ten maize cultivars showed that association between dendrogram obtained by RAPD marker and kernel colour. Shiv Narayan Sharma *et al.* (2009) evaluated the genetic diversity and relationship among *Andrographis paniculata* genotypes using RAPD. Out of 27 primers,
10 primers were found to generate clear and polymorphic bands. A total of 37 bands were generated from 10 primers. Of which 26 were polymorphic. The percentage of polymorphism was 70.27.

Random amplified polymorphic DNA markers were used to evaluate the genetic diversity in a representative population of *Jatropha curcas* L. from different eco-geographical regions of India. Out of 50 dacamer primers used, 44 yielded polymorphic banding pattern. A total of 328 DNA bands were obtained, of which 308 (93.90%) were polymorphic. The polymorphism was scored and used in band sharing analysis to identify genetic relationship. Cluster analysis based on Jaccard’s similarity coefficient using UPGMA grouped all the 40 genotypes into two major groups at a similarity coefficient of 0.54. Similarity indices ranged from 0.44 to 0.92 with an average of 0.73, indicating a moderate to high genetic variability among the genotypes (Ikbal *et al*., 2009). Arghavani *et al.* (2010) conducted a study to analyze genetic diversity in two *Agropyron* species, *A. pectiniforme* and *A. elongatum*, by RAPD analysis. Random Amplified Polymorphic DNA (RAPD) analysis using 12 primers produced 142 polymorphic bands with lengths ranging 564 to 2,000 bp. On the basis of Nei's gene index, the genetic diversity within ecotypes varied from 0.1014 to 0.178. The highest and lowest of this index were obtained in ecotype of 6951 (from *A. pectiniforme*; 0.178) and ecotype of 225 (from *A. elongatum*; 0.1014), respectively. The results showed high variation within ecotypes about two times higher than between ecotypes (31.24). Cluster analysis based on RAPD data using Nei's genetic distance categories the entries into four clusters. Using principle coordinate analysis, the first three coordinates accounted for the 52.84% of the total variation. Classifying the ecotypes by the two coordinates verified the results of cluster analysis.

### 3.3. MATERIALS AND METHODS

#### 3.3.1. Plant material

##### 3.3.1.1. Study species and population sampling

An extensive field survey was carried out throughout Kerala, South India and sixteen *Kaempferia galanga* accessions were collected from different geographical locations (Table 3.1, Plate 3.1, 3.2, 3.3.). Accessions are separated geographically by minimum distance of 40 kms. RAPD analysis was done for these 16 accessions of *K. galanga*.
3.3.2. DNA isolation

3.3.2.1. Preparation of Genomic DNA from Plant Tissue using CTAB

The application of molecular biology techniques to the analysis of the complex genomes depends on the ability to prepare pure, high molecular weight DNA. The modified method of Murray and Thompson, 1980 was used. It is based upon a series of treatments with a non ionic detergent (CTAB) to lyse the cells and purify the nucleic acid.

Principle

Cetyltrimethyl Ammonium Bromide (CTAB) based DNA isolation was initially used in bacteria and later modified to obtain DNA from plants (Murray and Thompson, 1980). CTAB forms an insoluble complex with nucleic acids when the initial NaCl concentration is lowered.

Critical parameters: The aim of any genomic DNA preparation technique is to isolate high-molecular weight DNA of sufficient purity. Two factors affect the size of the DNA isolated: shear forces and nuclease activity. As noted in the protocols, lysates should be treated gently to minimize shear forces. Plant cells are rich in nucleases. To reduce nuclease activity, the tissue should be frozen quickly and thawed only in the presence of an extraction buffer that contains detergent and a high concentration of EDTA.

Plant DNA isolation using the basic protocol should be in the range of 50 kb in length, which is quite acceptable for most applications. 1% polyvinylpyrrolidone (Mol. Wt. = 40,000; Sigma) was included during tissue homogenization to absorb the phenolic compounds.

3.3.2.2. DNA isolation Protocol

Step I (Day 1)

- 4 gms of fresh leaf samples were taken for the isolation of DNA
- The young leaf samples were cut into pieces using scissors and made into fine powder using Liquid Nitrogen (LN2) in a mortar and pestle.
- 1% β-mercaptoethanol was added to the extraction buffer and warmed at 65ºC for 5-10 min.
- 8-10 ml of warm extraction buffer was added to the ground sample approximately and 0.4 to 2.5 ml CTAB/NaCl solution was added for each gram of fresh leaf tissue and mixed to form a slurry.
The thoroughly mixed slurry was then transferred to a screw capped 30 ml centrifuge tube.

The tubes were incubated at 65°C for 1-2 hours with occasional mixing.

Equal amount of CHCl₃: isoamylalcohol mixture in 24:1 ratio was added to the slurry.

The mixture was centrifuged at 10,000 rpm for 5 min. at 4°C.

Clear (greenish yellow) supernatant was transferred to a fresh tube and double the volume of CTAB precipitation buffer was added.

Then it was incubated at 37°C overnight in a water bath.

**Step II (DAY 2)**

The incubated samples were centrifuged at 8000 rpm for 8-10 min. at 4°C.

The pellet was collected and supernatant was discarded.

1 ml of high salt TE was added to resuspend the pellet.

The suspended pellet was transferred 2 ml eppendorff tubs to glass tubes.

And 1.0 ml (0.6 volume) of isopropanol and incubate at -20°C for 30 min.

The tubes were centrifuged at 8000 rpm for 10 min. at 4°C.

The pellets were washed with 1 ml of 80% ethanol by spinning at 10,000 rpm for 5 min at 4°C.

The pellets were re suspended in 0.5 ml of 1 X TE.

The solution was transferred to 1.5 ml eppendorff tubes.

**RNase treatment**

2 µl of RNase stock solution (10 mg /ml) was added to the nucleic acid mixture in the eppendorff tube and incubated at 55°C for 10 min. or at 37°C for 1 hour.

Equal volume (0.5 ml) of phenol-chloroform and centrifuged was added at 10,000 rpm for 5 min.

The upper aqueous phase was collected and transferred into a fresh tube. The double the volume of 100% ethanol and 1/10th (50 µl) volume of 3 M sodium acetate was added.

The tubes were kept at -20°C over night for preparation.

**Step III (DAY 3)**

The precipitated samples were centrifuged at 12,000 rpm for 15 min. at 4°C.

The pellet was collected and washed with 0.5 ml of 70% ethanol.
Centrifugation was done at 10,000 rpm for 5 min. at 4°C.
The pellets were re suspended in 100µl 1X TE.
The re suspended pellets were collected and stored at -20°C.

3.3.3. Spectrophotometric estimation of nucleic acids
The amount of DNA/ RNA present in the pre-diluted sample was quantified using this technique. The absorbance of nucleic acid was read at 260 nm while that of protein was read at 280nm. The ratio of the absorbance at 260/280 was used to determine the purity of nucleic acid.

\[ \text{Ratio} = \frac{A_{260\text{nm}}}{A_{280\text{nm}}} \]

The optimum value was taken as 1.8 for pure DNA.

3.3.4. Sample preparation for UV spectrophotometry
To 10 µl of 1/100 diluted sample in 1X TE buffer 1.90ml of 1X TE Buffer was added. 2ml of the 1X TBE buffer in a quartz cuvette was taken to do baseline correction. The absorbance of the sample both at 260 nm and 280 nm was read and ratio was calculated. An absorbance (A_{260}) of 1.0 corresponds to 50 µg ds DNA / ml of the solution and from this the concentration of DNA was calculated.

3.3.5. PCR Assay for Randomly Amplified DNA fingerprinting
3.3.5.1. PCR reaction condition (Basic protocol)
The polymerase chain reaction was performed in a reaction volume of 25 µl containing 1x Taq DNA polymerase buffer with 1.5 mM MgCl₂, 0.2mM of dNTP’s, 1.0 U Taq DNA polymerase (Finnzymes, Helsinki, Finland)15 pmole random primer (IDT, Coraville, USA), 50 ng genomic DNA. Amplification was performed in a Thermal Cycler (MJ Research PTC- 100, Watertown, USA). The RAPD reaction was performed on the thermal cycler by the following parameters: Initial denaturation at 94 °C for 5 min, then 38 cycles of 94°C for 2 min, 37°C for 2 min, 72°C for 2 min, and final extension at 72°C for 7 min.

3.3.5.2. Electrophoresis
The PCR products (14 µl) were mixed with 6x gel loading buffer (2 µl) and loaded onto an agarose (1.4% w/v) gel electrophoresis in 0.5x TBE (Tris-Borate-EDTA) buffer at 100 V for 150 min. The gel was stained in ethidium bromide solution, visualized under UV in GEL DOC (UK) Image Analysis System with UViTEC analysis package (Cambridge, UK).
3.3.6. Scoring and analysis of RAPD data

Amplification with each random primer was repeated 2 times and those primers that produced reproducible and consistent bands were selected for data generation (Table 3.3). Reproducible RAPD products were scored as present (1) or absent (0) for a fragment. Bands of equal molecular mass and mobility generated by the same primer were considered as of identical locus. The distance matrix was formulated by Nei’s genetic distance analysis methods (1972) and the phenogram constructed using the using the POPGENE 32 software. The normalized statistical methods of Nei were taken for determining the level of association between the matrices. The variance components were tested statistically by nonparametric randomization tests using 1,000 permutations. A UPGMA (unweighted pair –group method using arithmetic average) dendrogram was constructed based on the matrix of Nei’s genetic identity using the Modified NEIGHBOR procedure of PHYLIP (Phylogeny Inference Package) version 3.75c, Felsenstein (1993). Free tree- Freeware program (Pavlicek et al. 1999) was used to generate Nei (1972) standard genetic distances between regions for bootstrap analysis and the generated trees were viewed using Tree View (Win 32) 1.6.6 program. The tree was exported as neckwick file (nwk) and viewed in Mega 4.0 for branch swapping and modification of tree for interpretation.

3.4. Results and discussion

3.4.1. Primers used for RAPD and amplification

After optimization of the amplification conditions, DNA of 16 *Kaempferia galanga* accessions was amplified with 15 different decamer random primers (Table 3.2). A total of 109 DNA fragments were generated by the 12 primers with an average of about 6.2 bands per primer.

Reactions were duplicated to check the consistency of the amplified products. Only, easily resolved bright DNA bands were considered to be present and scored.

3.4.2. Polymorphism as revealed by RAPD markers

Approximately 86.1% polymorphisms estimated from 93 of 109 fragments were polymorphic with 12 primers used among the 16 *Kaempferia galanga* accessions. The other 16 bands were monomorphic in the 16 accessions. In the present study, the 16 accessions appeared to show differences/variability with the 12 primers
used. Among 12 primers S63 individually was so informative as to differentiate all the accessions. Other highly polymorphic profiles were obtained with the primers such as S61, S63, S64, S73 and S80.

3.4.3. Genetic distances between the accessions

The genetic distance for RAPD data using 16 accessions was constructed according to Nei (1978), as shown in Table 3.4, 3.5, and relationships between accessions were presented graphically in the form of a dendrogram in Figure 3.2. The genetic distance between the accessions ranged from 0.1 to 0.85. The lowest genetic distance of 0.1 was seen in genotypes KGL13 and KGL14. The genetic identity ranged from 0.43 to 0.94. The genotypes KGL09, KGL11 and KGL13 were the second similar group with a genetic distance of 0.11. The most dissimilar of all the accessions were KGL04 and KGL15 with a genetic distance of 0.85. The overall observed and effective number of alleles is about 1.89 and 1.45 respectively. Nei (1978) overall gene diversity is 0.2747. The level of polymorphism observed in the present study was fairly high, indicating a wide and diverse genetic base. This type of genetic variation is also observed in other Zingiberaceae members like Alpinia calcarata (Regha and John, 2008), Zingiber zerumbet (Kavitha et al., 2010). Since most zingiberous members are propagated through rhizomes and propagation through seeds are seldom, variation could have occurred only by external environmental pressures such as radiation and physiological stress viz. the nature of soil (type, PH etc.).

3.4.4. Clustering pattern

The cluster analysis based on genetic distance values classified all the Kaempferia galanga accessions into two major clusters/groups I and II (Figure 3.1). Group I includes only three genotypes KGL01, KGL02 and KGL03 from Kasargoad and Kannur districts i.e North Kerala (Plate 3.3) which were highly different from other genotypes. These genotypes are wild varieties. The remaining genotypes form group II. Within the group II there are three subgroups. KGL09, KGL10, KGL11 KGL12, KGL13 and KGL14 are grouped into subgroup IIA. All these accessions are from Trissur, Ernakulam, Iduki and Kottayam i.e Central and South Kerala (Plate 3.3). KGL06, KGL07, KGL08, KGL15 and KGL16 form subgroup IIB. This group
contains accessions from widely different areas like Malapuram, Palakkad, Trissur and Trivandrum. This constitutes both North, Central and South Kerala. All the accessions in group IIB were commercially cultivated varieties in large scale. KGL06 and KGL07 were cultivated by Kottakal aryavhyasala (Ayurveda sala), Kottakal, Malapuram Dt. KGL08 and KGL16 were cultivated in Kerala Agricultural University Vellanikkara, Trissur Dt. and Tropical Botanical Garden Palode, Trivandrum Dt. respectively. KGL11 is also a commercial cultivated variety collected from Kothamangalam (Ernakulam Dt.) which is different from other varieties. This particular variety is cultivated and maintained by farmers of Central Kerala. In Kerala K. galanga is cultivated mainly for oil extraction and Ayurvedic preparations. Phytochemical analysis was done for KGL08 (refer chapter IV). This work provides a scope to do detailed comparative phytochemical studies on varieties identified by RAPD and further conserve the elite variety through appropriate propagation methods. Thus RAPD proves its potentiality in identifying elite varieties. KGL04 and KGL05 stand separately as subgroup IIC. These two accessions were collected from Kozhikode and Wayanad (North Kerala). There is wide genetic diversity that cannot be interpreted based on the geographical isolation (i.e Lowland, Midland and Highland). Since there were not much of differences in morphology of these accessions except leaf size and length of the leaf-sheath (Plate 3.1, 3.2). Molecular analysis had proved valuable in separating these accessions where much of genetic variations have occurred between these accessions which is evidently shown in the distance matrix (Table 3.4). This study also insists the need to conserve the elite clone for breeding purpose through tissue culture. KGL08 was assumed to be the elite clone since it is one of the cultivated varieties in Kerala Agricultural University, Vellanikkara, Trissur Dt. It is characterized by large leaves and dense rhizomes. This particular clone is propagated through tissue culture (refer Chapter II). Antimicrobial activity is discussed in Chapter V and Antioxidant activity in chapter VI.