INTRODUCTION
1. INTRODUCTION

Spices have had a profound influence in the course of history and civilization. Spices and precious metals hold a high and very special place in the annals of exploration in the middle ages and the Renaissance. The majority of spices originated in the Asiatic tropics and they were among the first objects of commerce between the east and the west. Spices have played an important role in the history of civilization, exploration and commerce. So great was the value of spices in ancient and medieval times that they are often equated with gold and precious stones.

Spices, which have played an important role in the history of exploration and development, are no longer luxury items of great cost. With the advent of refrigeration, there is less demand in the west to preserve and flavour foods at home, but they are widely used by the meat, sauce, canning, frozen food industries, food manufacturing industry generally. They are also used in the cosmetic and perfumery industries, including its use in soap and toothpaste. Spices, or their essential oil, are of some importance in the preparation of liqueurs and cordials. They are also used in various ayurvedic and allopathic medicine. Bakers use it liberally in cookies and in hot drinks. *Cinnamomum* and *Cassia* are said to be among the oldest spices. *Cinnamomum* has fragrant, sweet and warm taste.

1.1 *Cinnamomum Schaeffer* (Lauraceae)

Local name – bojewar, dalchini, tej, tamalapatra

English name: Ceylon Cinnamon, Cinnamon.

The cinnamon of the market are the inner barks obtained from trees of tropical countries and islands. Cinnamon is an old tree, a highly prized spice whose bark is widely used as a spice. It is principally employed in cookery as a spice and flavouring agent. *Cinnamomum* stands out of all spices in its “warmth” and ranks as second to pepper which is often used for food flavouring in North American Kitchens. As spices, cinnamon and Cassia stand first as commercially important commodities and cinnamon is still considered one of the finest sweet spices. It is indigenous in Sri Lanka, which still produces the largest quantity and best quality, mainly in the form of quills.
Cinnamomum species, are commercially valuable source of camphor, cinnamaldehyde and safrol oil in the world. Cinnamomum is the largest genus in the Lauraceae family and comprises 250 [201] species, which are distributed in India, Sri Lanka and Australia. Cinnamomum was first introduced in India from Sri Lanka. 13 species of Cinnamomum have been found in south India [105]. Many species of cinnamon yield a volatile oil on distillation. The most important cinnamon oil in world trade are those from C.zeylanicum Bl., C.cassia Presl. and C.camphora (L.) Presl. However, a number of other Cinnamomum species are utilized as sources of oils and distilled on a much smaller scale and the oils are used both locally or exported [173]. The dried bark contains essential oil, fixed oil, tannin, resin, proteins, cellulose, pentosans, mucilage, starch and minerals, while the presence of calcium oxalate crystals indicate low quality bark.

Analysis of four samples of Indian bark gave the following average proportions of trace elements, in parts per million, K 1590, Ca 1091, Ti<40, Cr<35, Mn 179, Fe 89, Zn<25, Rb 30, Sr 57. Essential oil content of bark ranges between 0.5 and 2.0%. The main constituent is cinnamaldehyde at 60-70% oil distilled from outer and inner bark differs and only inner bark (quills) is normally distilled for oil to be used as spice [65]. Chemical composition of essential oil include ethyl cinnamate, eugenol, cinnamaldehyde, beta caryophyllene, linalool and methyl chavical.

Cinnamomum leaf oil

Leaf oil is a yellow to brownish yellow liquid with a warm, spicy and somewhat harsh odour, lacking in the richness of bark oil. Its taste is slightly bitter, burning, very spicy and powerful. Eugenol content of Sri Lankan leaf oil ranges 60-85% depending on the place of origin. Seychelles leaf oil is a valued source of eugenol usually above 90% with phenols 78-95% and aldehydes 5%. Major uses of leaf oil is in food processing and pharmaceutical industries.

Oleoresin

Oleoresin is a deep reddish or greenish brown rather viscous liquid, with a volatile oil content of 16-65%. This is usually prepared by extracting cinnamon bark with organic solvents. Oleoresin is used in flavouring cake and similar mixes, pickles, prepared meats, convenience foods and related products where flavour stability at
high temperature is important. It is principally employed in cookery as a condiment and flavouring material. It is used in the preparation of chocolate, especially in Mexico, which is the main importer of true cinnamon. It is also used in the preparation of some kinds of desserts, such as apple pie, donuts, and cinnamon buns as well as spicy candies, tea, hot cocoa, and liqueurs. True cinnamon, rather than cassia, is more suitable for use in sweet dishes. In the Middle East, it is often used in savoury dishes of chicken and lamb. In the United States, cinnamon and sugar are often used to flavour cereals, bread-based dishes, and fruits, especially apples, a cinnamon-sugar mixture is even sold separately for such purposes. Cinnamon can also be used in pickling. Cinnamon bark is one of the few spices that can be consumed directly. It is often mixed with rosewater or other spices to make a cinnamon-based curry powder for stews. Its flavour is due to an aromatic essential oil. Cinnamon is used as an alternative to traditional food preservatives. It is used in both sweet and savoury foods. Cinnamon could be added to various drinks, including tea and milkshakes to make it more palatable.

Cinnamomum is carminative, anti-infective, antifungal, digestive aid. Spices have been used from ancient times to increase metabolism, raise body heat (thermogenesis), improve digestion and potentiate the effects of other substances. The bark of the Cinnamomum which is acrid, bitter, sweet and aromatic is reported to be used in treating bronchitis, asthma, cephalagia, uropathy, nausea, vomiting, flatulence, fever and for restoring normal skin colour. Cinnamon oil is stomachic and carminative. It is reported to be useful in anorexia inflammations, and tubercular ulcers. Regarding sugar metabolism a study by the United States Department of Agriculture (USDA) looked at the potential effects of 49 spices on insulin function. The researchers found that cinnamon was the most bioactive in directly stimulating cellular glucose metabolism i.e., the ability of cells to utilize sugar, the effects of cinnamon for potentiating insulin.

Cinnamomum flavour is due to an aromatic essential oil extracted from stem bark that makes up 0.5 to 1 % of its composition (oil is of golden yellow color) [65], [162], [165] with the characteristic odour of cinnamon and a very hot aromatic taste. The pungent taste and scent come from cinnamic aldehyde or cinnamaldehyde. Other chemical components of the essential oil include ethyl
cinnamate, eugenol (found mostly in leaves), beta-caryophyllene, linalool and methyl chavicol. In medicine, also it acts like other volatile oils and once it had a reputation as a cure for cold. It has also been used to treat diarrhoea and other problems of the digestive system. The essential oil of cinnamon has antimicrobial and antioxidant properties, which can aid in the preservation of certain foods. *Cinnamomum* powder is used in bladder infection, to strengthen the immune system and in indigestion. Oil is extracted from three parts of the tree. The oil from the bark is the common essential oil of cinnamon, it is brown and viscid. The oil from the leaves contain 70-80% eugenol with odour of clove oil and is often known as clove oil. *Cinnamomum* is also used in Ayurvedic and traditional Chinese medicine for its hypoglycaemic, digestive, antispasmodic and antiseptic properties. Essential oils are used in classification and phylogenetic studies of the genus *Cinnamomum* [53].

Pharmacological experiments suggest that the cinnamon-derived dietary factor cinnamic aldehyde (cinnamaldehyde) activates the Nrf2-dependent antioxidant response in human epithelial colon cells and may, therefore, represent an experimental chemopreventive dietary factor targeting colorectal carcinogenesis. Cinnamon has been reported to have remarkable pharmacological effects in the treatment of Type 2 diabetes mellitus and insulin resistance. Recent advancement in phytochemistry has shown that it is a cinnamotannin B1 isolated from *Cinnamomum zeylanicum* which is of therapeutic effect on Type 2 diabetes. The bark of *C.zeylanicum* was found to control diabetes through its active polymer compound methylhydroxy chalcone (MHCP) [76]. MHCP increases the conversion of glucose to energy and also blocks the formation of dangerous free radicals. Squeezing out free radical activity reduces the progression of diabetic complications. The cinnamon polymers assist diabetes in two important ways: firstly, the substance disables certain enzymes that cause insulin resistances and may help type 2 conditions. Secondly ingesting this compound, increases sensitivity to insulin and effectively distributes insulin in the body. This polymer has been patented and available as the water soluble extract of the compound cinnulin PF. The polymer helps to decrease patients total cholesterol up to 26%. The bad cholesterol is lowered by 10 to 24%. Also cinnamon has been proposed for use as an insect repellent, although it remains untested. cinnamon leaf oil has been found to be very effective in killing mosquito
larvae. The compounds cinnamaldehyde, cinnamyl acetate, eugenol, and anethole, that are contained in cinnamon leaf oil, were found to have the highest effectiveness against mosquito larvae.

It is reported that drinking tea regularly made from the bark of Sri Lankan cinnamon could be beneficial to oxidative stress related illness in humans, as the plant part contains significant antibacterial and antioxidant potential [17], [18]. Approximately, 60-80% of the world population still relies on traditional medicines for treatment of common illness, (World Health Organisation) [192], [200]. Traditional remedies have a long-standing history in many locations and applicable tools for treating ailments.

1.1.1 Cytogenetics

_Cinnamomum verum_ J.S. Presl (Syn _C. zeylanicum_ Blume; _Laurus cinnamomum_ L.) is frequently referred in the literature by its synonym _C. zeylanicum_. The generic name is derived from the Arabic or Persian name via the Greek, Amomum meaning spice, and the prefix chini to its believed origin. It is known commercially as true cinnamon to distinguish it and its products from those derived from other _Cinnamomum_ species. In India, as dalchini, in Sanskrit as tamalpatra.

The genus _Cinnamomum_ Schaeffer, belongs to the family Lauraceae. They are mainly evergreen trees of the tropics and subtropics. Willies states that there are about 250 species of _Cinnamomum_, Bailey says more than 50 species, while Brown says about 100, Kostermans [105] lists 462 binomials, some of which, of course, are synonyms. They are evergreen trees and shrubs found in south eastern and eastern Asia, through Malaysia to Australia and the Pacific. As pointed out by Burkill, all the species are aromatic, the aroma depending on different substances and their mixtures. _Cinnamomum zeylanicum_ Bl. (Syn _C. verum_ Presl) is the true cinnamon of commerce.

Other species yielding spices are _C. cassia_ Presl, Chinese cassia, _C. burmanni_, (C.G. and Th. Nees) Bl., Indonesian or padang cassia and _C. loureirii_, Nees, Saigon cassia or cinnamon. In addition, there is _C. tamala_ (Buch.-Ham) Th. Nees and Eberm., which gives Indian cassia, and which according to Sastri, it is the source of tejpat leaves, which are used extensively as a spice in northern India. Camphor oil is
obtained by distilling the wood or leaves of *Cinnamomum camphora*. Other species of *Cinnamomum*, whose barks are used as spice or in medicine include *C. culilawan* (Roxb.) Presl. from moluccas, *C.iners* Reinw., which is available in Western India and Tenasserim (Burma) to Malaysia, Indonesia and the Philippines, *C.javanicum* B1. Serim and Western Malaysia and *C.sintoc* Bl. in Java.

**Species of Cinnamomum**

There are 250 species of *Cinnamomum* which are distributed in tropical subtropical East Asia, Australia and Pacific Islands [201]. There are 13 South Indian species of *Cinnamomum* [105].

Somatic chromosome number of 2n=24 has been determined. Gametic chromosome number of X=12 has been reported by Darlington and Wylie, and the somatic diploid number of *C.zeylanicum* and some of the other species as 2n=24. The importance of polyploidy in the genetic improvement of tree species has been well emphasized by Gotafsson [62] and Martin [117].

**1.1.2 Random Amplified Polymorphic DNA fingerprinting**

Genetic variation is a prerequisite for any crop improvement programme. Diversity analysis is traditionally done based on differences in morphological characteristics which is mostly influenced by environmental factors. Studies based on seed proteins and isoenzymes are not so efficient owing to lack of polymorphism as well as being influenced by environment, source tissue and plant development stages. In recent years, limitations of morphological and biochemical markers has been overcome by molecular markers. The extent of genetic variation among individuals or different populations depends upon several factors, including gene flow between populations and various other factors. Among the different molecular markers, some are relatively cheaper, and simple to use in a variety of applications in plant research. One of such markers is Random Amplified Polymorphic DNA (RAPD), and is one of the Polymerase Chain Reaction (PCR) based DNA marker, defined as an assay based on the amplification of genomic DNA with single primer of arbitrary nucleotide sequence. Usual PCR based DNA markers have been handy and convenient alternative techniques for investigations of genetic variation and genome mapping. These markers can be used in studying genetic diversity, varietal identification, etc.
The information on polymorphism using RAPD in a set of genotypes, is useful in tagging genes of interest and genetic mapping in long run to facilitate marker assisted selection. RAPD analysis samples the genome more randomly than other methods and has been successfully employed in the construction of linkage maps. Being simple and non-radioactive, the technique is quite sensitive and is also used to detect genetic variation in several living things. It has been extensively used for molecular fingerprinting and population diversity analysis, as in Cassia [7], Sunflower [85], etc. The variations that can be accounted for between and within populations through RAPD appear to be numerous. To take right measures to protect wild resources of Cinnamomum zeylanicum, it is important to study the range of genetic variation, genetic structure, diversification trend and other factors affecting genetic structure of populations of this important species.

Genetic markers are powerful tools for evolutionary and population studies, constructing genetic maps, and managing applied breeding programmes. When the genetic marker data is interpreted correctly, these are valuable for examining genetic variation among and within populations, assessing levels of outcrossing and inbreeding and genetic identification or fingerprinting of varieties or pedigrees. Genetic marker data can also be used for assisting with early selection of better genotypes, rather than waiting for the tree to express the trait much later [195], [109].

Genetic polymorphism is classically defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variations or genotypes [93]. Various types of genetic markers are utilized to evaluate DNA polymorphism and are generally classified as hybridization based markers and polymerase chain reaction (PCR) based markers. The widely used hybridization based marker is Restriction Fragment Length Polymorphism (RFLP) and PCR based marker is Random Amplified Polymorphic DNA (RAPD).

In RFLP analysis, restriction digest of genomic DNA is resolved by gel electrophoresis and then blotted on to a nitrocellulose membrane to provide the stationary phase required for nucleic acid hybridization. Specific banding patterns are then visualized by hybridization with labelled probes. RFLP utility has been hampered due to the large amount of DNA required for restriction digestion and southern blotting. The requirement of radioactive isotope makes the analysis
relatively expensive and hazardous. The assay is time consuming and labour intensive, and only one out of several marker may be polymorphic. Their inability to detect single base changes restricts their use in detecting point mutations occurring within the regions at which they are detecting polymorphism [93].

Random amplified polymorphic DNA (RAPD) was developed by Williams [189] and as arbitrarily primed PCR (AP-PCR) by Welsh McClelland [186]. In RAPD analysis, several loci or specific nucleotide sequences of genomic DNA are amplified in vitro by single stranded short decamer deoxynucleotide primers via Polymerase Chain Reaction with thermostable DNA polymerase. Polymorphism is normally indicated by the presence, or absence, of an amplified product from a single locus [185], [145], [139].

RAPD marker behaves as a dominant genetic marker and segregates in a mendelian fashion [185], [145], [175]. The presence of RAPD band corresponds to the dominant allele, where as its absence denotes recessive allele. Thus, heterozygous and homzygous dominant individuals cannot be differentiated with RAPD markers. In F2 these individuals would segregate in 3:1 ratio [190].

In recent years, many variants of RAPD assay have been developed and these are Sequence Characterised Amplified Regions (SCARS), Cleved Amplified Polymorphic Sequences (CAPS), Randomly Amplified Microsatellite Polymorphism (RAMPO), Amplified Fragment Length Polymorphism (AFLP), DNA Amplification Finger Printing (DAF), Arbitrary Primed Polymerase Chain Reaction (AP-PCR) and Sequence Tagged Sites (STS) [93], SSR, ISSR, SNP, RAPD markers are widely used due to simplicity of technique as compared to RFLP. One of the key advantages of RAPD analysis is the lack of dependence on large quantities of relatively pure genomic DNA. This method requires nanogram quantity of genomic DNA and does not involve blotting or radioactive probes. The extent of genetic polymorphism detected by RAPD is greater than RFLP [191]. RAPD and its variants have been extensively used in characterization of genetic diversity, population genetic structure [199] varietal identification, genetic relationships, phylogeny and evolution, marker assisted breeding, gene mapping, identification of sex in plants, taxonomic studies, identification of natural hybrids, characterization of somaclonal variations. RAPD and
Genetic diversity provides the template for adaptation and evolution of populations and species. It is essential to the long term survival of tree species. Without it, there may be a risk of its extinction because of lack of adaptive ability [74]. The patterns of genetic variability among populations can be influenced by mutation, genetic drift, mating system, gene flow and selection [167], [120]. Measurement of pattern and extent of genetic variability in natural populations of various species is a major thrust area of population genetics and plant breeding. Out crossing species tend to have higher levels of variability within populations, but there is smaller degree of differentiation among populations than among self pollinated species [73], [161].

Randomly amplified polymorphic DNA (RAPD) analysis via the polymerase chain reaction (PCR) has profoundly increased the potential to easily detect genetic polymorphism among organisms, particularly for those in which DNA sequence information is unknown [189], [68]. In recent years, there has been increased interest in the use of DNA based markers for a variety of applications in population genetics, conservation and tree improvement. RAPDs have been recently used to analyze genetic variation in Eucalyptus microtheca populations [112], Andrographis paniculata [134], quantify intraspecific genetic variation in Swietenia macrophylla populations [60], quantify genetic diversity in spruce [21] and quantify genetic variation in other native plants and crop species [83].

Genetic diversity has also been extensively studied by using RAPD markers in Indian tomato cultivars [6], upland cotton varieties [114], Oryza malampuzhaensis [174], Vigna subterranean [5], Poplar [147], Ranunculus reptans [49], Indian tetraploid wheats [143], Elymus caninus [171], Cyanodon [10], Chaemonomeles [16], Lilium martagon [140], Passiflora [41], Digitalis obscura [125], Cymbipodium cultivars [130], Bertholletia excels [95], Panax quiniquifolius [33], Indian mustard [87], Mahogony [27], Buffalo grass [83], [22], Phyllanthus amarus [127], Amaranthus [28], [46], Lettuce [135], Cotton [137], Eucalyptus [36], Azadirachta indica [37], Ziziphus sp.[40], Cassia [58], Urginea indica [75], Rosmarinus tomentosus [94], Zingiber officinale [96], Mentha sp. [98], Tomato
Varietal identification of cultivated plants based on morphological traits, like flower color or grain colour, etc., would necessarily require the plant to grow to a flowering/fruiting stage. RAPD fingerprints can characterize and identify the variety or genotype at faster rate without waiting for the plant to reach flowering or fruiting stage. Varietal identification can be important for protecting Intellectual Property Rights. RAPD fingerprinting have been recently used for varietal identification of 7 japonica and two Indian rice varieties [123], fifty-one strawberry cultivars of North America could be identified by RAPD fingerprinting [35], varietal identification based on RAPD bands have been done in Pearl millet [29], Rose [14], Pigeon pea [149], Sweet potato [31], Apple cultivars [104], Celery cultivars [197], Rice accessions [54], Cacao cultivars [185], Alysicarpus [92], Sunflower hybrids [85]. An understanding of genetic relationships between cultivated and wild species is an important component of plant breeding program. DNA marker like RAPD provides an efficient method to estimate genome relations among wild and cultivated taxon, [84] analyzed seventy five accessions belonging to 14 species of the genus Cicer with PCR based molecular markers to determine their phylogenetic relationships. RAPD markers have been used to establish the species relationships in Fagopyrum [163], to study genetic relationships among nine annual Cicer species [2], Hordeum [45], [3], [152], Chenopodium [155], Alfalfa [55], Avocado [48], Paspalum [90], Eleusine [78], Panicum [116], Echinochloa [77] and other taxa. RAPD analysis has been successfully used to generate complete genomic maps and linkage studies in several plant species, like Arabidopsis [150], Maize [146], Eucalyptus grandis and E.urophylla [63], Helianthes anomalous [151], Medicago sativa [101], [103], Oat [129], Rice [108], Brassica nigra [178], Celery [198], Citrus [25], Viciafaba [177], Betula alleghaniesis [154], Lolium, Festuca and Vulpia [23] and other plant species. RAPD analysis is also being employed in various taxonomic studies.
Autumn buttercup *Ranunculus acriformis* var. *aestivalis*, an endangered endemic plant in USA, is treated differently by various taxonomists [24] who made an RAPD analysis of *R. acriformis* var. *aestivalis* and elevated it to a specific rank as *Ranunculus aestivalis* (Benson.) Buren and Harper. Wilkie [188] analyzed RAPD pattern in six *Allium* species, including cultivated *A. cepa* and confirmed broadly the previous taxonomic treatment based on morphology. However, *A. roybi* previously placed in section *Rhizirideum*, is not supported by RAPD analysis. This study suggests that *A. roybi* would be more appropriately classified within section, *Cepa*. Several such examples of RAPD application in taxonomy are available in literature.

RAPD analysis has been extensively used to identify RAPD markers linked to disease resistance, stress tolerance, seed quality, insect resistance, nematode tolerance, cold acclimatization, [139] etc. Breeder, instead of selecting for a trait directly can select a co-segregating RAPD marker linked to desirable gene, eg., disease resistant gene. In common bean, RAPD markers were identified linked to dominant gene (Arc) containing resistance to *anthracnose* [1], *Uromyces appendiculatus* [69], and *Cucumis* [115]. In tomato RAPD markers for fusarium crown and root rot resistant gene (Frl) [47]. In apples, RAPD marker have been identified as linked to resistance gene Vfl [196]. Thus RAPD marker assisted breeding holds a promising future. Among the methods used in studies, DNA markers have proved to be excellent parameter to resolve the problems of identification of critical taxa and to understand their relationships and taxonomic status. Somaclonal variations have been characterized using RAPD and its variant methods in *Rice* [29], *Brassica napus* [141], *Gerbera* [88].

Sex in dioecious plants at seeding stage have been successfully identified by using RAPD. *Pistacia vera* or Pistachio trees are cultivated for nuts. Sex of the plants can be recognized only when the trees are 4-8 year old. Method to determine gender of the plants at seedling stage would facilitate breeding and planting programme. Hormaza and Polito [81] used 700 decamer primers and one primer OPO-08 produced 945 bp marker linked to female plant. This marker is absent in all the male plants. Such markers also helped in sex determination of *Asparagus* [131], *Momordica dioica* [15], *Carica papaya* [61], *Salix viminalis* [66] etc.
RAPD-PCR based SCAR markers were used for genetic characterization and authentication of medicinal and other plant species. RAPD-SCAR markers were used for authentication of *Phyllanthus emblica* [184], *Bentgrass* [44], *Embelia ribes* [38], *Pueraria tuberosa* [39], etc.

The present study was undertaken to investigate the magnitude of genetic diversity at molecular level by using RAPD markers. In India, the application and use of the technique is very recent. Hence, the aim of the present investigation was to analyze the genetic diversity in *Cinnamomum zeylanicum* genotypes and to establish genetic relationships between *Cinnamomum* species using RAPD markers.

**2. OBJECTIVES OF THE STUDY**

- To study the distribution of *Cinnamomum* species in South India.
- To study the morphological diversity in the genus *Cinnamomum* distributed in Southern India.
- To assess the genetic diversity in *C. zeylanicum* genotypes by using PCR based RAPD markers.
- To establish the genetic relationships among various species of *Cinnamomum* by using PCR based RAPD markers.
- Development of SCAR marker for *C. malabatrum* for authentication.