DISCUSSION
5.1 *Cinnamomum* and its importance

*Cinnamomum* is a warming, stimulating, pleasant-tasting plant with many uses. Cinnamon is widely used as a flavouring agent in candy, toothpaste, mouth fresheners etc. In herbal teas, cinnamon improves the flavour of less palatable herbs. But cinnamon has strong herbal remedial uses as well. In addition to having a germicidal effect, cinnamon helps to improve blood circulation and relieve discomfort in the abdomen. Cinnamon is more than just an everyday spice. Although cinnamon is popularly used in the flavouring industries, cosmetics and perfume preparations, it is also known for its extensive curative properties. It is used to cure several health complaints such as arthritis, bladder infections, skin disorders, diabetes etc.

5.2 RAPD Fingerprinting

5.3 Strengths and weaknesses of RAPD Primers

RAPD analysis make use of a set of PCR primers of 8-10 nucleotides whose sequence is essentially random. The random primers are tried individually or in pairs in PCR reactions to amplify fragments of genomic DNA from the organisms of interest. In most of the organisms, it is usually straightforward to identify a large number of RAPDs that can serve as genetic markers for many different kinds of genetic studies. Many amplified bands are typically observed for each primer set, but only some of these are polymorphic. The amplified bands that are not polymorphic are said to be monomorphic in the sample, which means that they are same from one individual to the next. One limitation of southern blotting is that it requires probes available in the form of cloned DNA and another limitation of PCR is that it requires sequence information.

5.4 Use of RAPD primers

Molecular primers have been used extensively in studying genetic diversity, genetic relationships and germplasm management. However, understanding of genetic variation between and within populations and how it is partitioned on the basis of geographic origin, is crucial as this helps to improve sampling efficiency [99], [166], [42]. 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]. RAPD fingerprints can characterize and identify the variety or genotype at faster rate without waiting for the plant to reach flowering or fruiting stage.

RAPD and its variants have been extensively used in characterization of genetic diversity, population genetic structure, 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 etc. RAPD and various applications have been summarized by Gresensoff [64], Newbury [128], Penner [139], Joshi [93]. 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. RAPD’s have been recently used to analyze genetic variation in Eucalyptus microtheca populations [112], quantify intraspecific genetic variation in Swietenia macrophylla populations [60], quantify genetic diversity in spruce [21].

Genetic diversity has also been extensively studied by using RAPD markers in Indian Tomoto cultivars [6], upland Cotton varieties [114], Oryza malampuzhaensis [174], Vigna subterranean [5], Poplar [147], Rannunculus reptans [49], Buffalo grass [83], [22], Phyllanthus amarus [127], Amaranthus [28], [46], Lettuce [135], Cotton [137], Eucalyptus [36], Adadirachta indica [37], Ziziphus sp.[40], Cassia [58], Urginea indica [75], and Triticum aestivum [176].

5.5 Intraspecific genetic diversity study

Intraspecific molecular diversity among the genotypes of Cinnamomum zeylanicum by using RAPD markers provides genetic data and a theoretical basis for protection of the Cinnamomum species. Present study shows how variation is
partitioned within and between different geographic locations by using molecular markers [99]. Frankel [51] opined that genetic variation is essential for long term survival of species and it is a critical feature in conservation. Ex-situ conservation will help to maintain the population of the red listed species by facilitating release back to nature for native habitat restoration [113]. Therefore, tracing successfully adapted variants at genetic level of Cinnamomum zeylanicum is of immediate necessity for their long-term preservation of these species.

For efficient conservation and management, the genetic composition of the species in different geographic locations needs to be assessed. Due to technical simplicity and speed, RAPD methodology has been used for diversity analysis in many red listed plants [113], [52], [132], [133]. The biodiversity study unfolds the quantum of variation prevailing in a given species per unit area, which helps to devise appropriate conservation strategies for future. PCR based RAPD marker was widely used in assessing genetic variation within species by measuring genetic diversity in many species including medicinal plants [134], [82], [179], [89]. Grouping of genotypes to their respective geographical locations and their individual identification with diversity analysis using molecular markers (RAPD) are the main emphasis of the study.

5.6 Intraspecific genetic diversity in Cinnamomum zeylanicum genotypes

PCR analysis using random decamer primers revealed significant differences in RAPD profiles in C.zeylanicum genotypes derived from different geographical locations. It has also detected small differences between genotypes. This study on C.zeylanicum deals with the molecular basis of genetic diversity by using RAPD markers. RAPD survey of the south Indian populations of C.zeylanicum demonstrated that, this ancient spice and medicinally important plant displays high levels of genetic variability.

89% of diversity was maintained between genotypes of C.zeylanicum. This finding is in agreement with the observation that out crossing plants retain considerable variability and that most variation is exhibited within populations [70]. Similar results were reported in other out crossing species such as Buchloe dactyloides [83], Populus tremuloides [199], Swietenia macrophylla [60], Eucalyptus
5.7 RAPD data analysis

RAPD data analysis of 15 genotypes of *C. zeylanicum* revealed 89% of polymorphism. A total of 133 bands were scored for 11 RAPD primers of which 119 bands are polymorphic with number of amplified bands ranging from 9 to 16, corresponding to an average of 12 bands per primer (Table-6). The level of genetic similarity between genotypes ranged from 0.32 to 0.87. The minimum genetic similarity of 0.32 is exhibited between genotypes CZ-455 (UAS, Dharwad) and CZ-465 (Bangalore) and maximum genetic similarity of 0.87 between genotypes 452 (Madikeri) and 456 (Yellapur). Maximum number of polymorphic (15) bands were obtained from OPB-17 and minimum number of 7 bands with OPB-11 and OPA-4 respectively (Table-6). Dendrogram (Fig-24) was drawn, based on the SI (Similarity Index) from RAPD data (Table-7). The Principal Component Analysis (PCA) supports major clustering pattern (Fig-25).

The genetic differentiation between genotypes of *C. zeylanicum* could broadly be explained as a result of abiotic (geographical e.g. hydrographic connections or climatic differentiation e.g. annual rainfall differences) and biotic (pollination between populations and seed dispersal) factors [91]. The percentage of polymorphism, i.e., 89% was higher in comparison to other endangered plants, like *Cathaya argyrophylla* (32%) [183], *Paeonia rockii* (27.6%), *Paeonia suffruticosa* (22.5%) [138], *Dacydium pierrei* (33.3%) [169], vulnerable medicinal plant *Oroxylum indicum* (49.6%) [91], *Costus speciosus* (35%) [9], *Lactoris fernandeziana* (Lactoridaceae) (24.5%) [20], etc. This shows that species genetic diversity is high.

High diversity in *C. zeylanicum* genotypes might be because of cross pollination by insects, dispersal of seeds, habitat changes and larger population size in different locations of Karnataka and other south Indian states. The significant degree of variation (Similarity Index 0.32) between genotypes CZ-440 and CZ-456 reveals maximum genetic diversity, which may be because of geographical isolation and change in the environmental conditions. There is a close genetic similarity of
87% between the genotypes CZ-456 and CZ-452, which clearly depict that genetically they are similar and it may be because of the similar environmental conditions.

In essence, the RAPD method used in this study displayed appreciable genetic variation or molecular polymorphism, which pre-existing in different collections of *C. zeylanicum*. In spite of their morphological identity, substantial genetic polymorphism was observed among the genotypes under study. The RAPD profiles display vast genetic variation which is the indicative of the evolving nature of the taxa. The study revealed that though the decamer primers are small in comparison to the large genome of *C. zeylanicum*, they produced appreciable amplicons, sufficient to demarcate all genotypes collected from the 15 locations (Table-1). The dendrogram also established genetic relatedness among different genotypes and quantum of changes that occurred in the genome in the course of evolution. The study confirms the suitability of RAPD as a reliable, simple, easy to handle and elegant tool in molecular diagnosis of different genotypes of spice and an important medicinal plant species available in the western ghats of Karnataka and other regions. Thus RAPD proved to be useful in molecular profiling of different genotypes of *C. zeylanicum* collected from diverse places. Currently, it is also proved that the entries, that were found to be similar in taxonomical classification based on morphological characters, do have divergence at the DNA level. Tracing successfully adapted variants at the genetic level of *C. zeylanicum* is of immediate necessity for their long-term preservation of these species.

The results of the present study on population genetic structure of *Cinnamomum zeylanicum* would help in designing methods for the collection and conservation. Accurate estimates of diversity are a prerequisite for optimizing sampling strategies and for conserving tree genetic resources. The genetic diversity revealed by RAPD is in agreement with the conclusion that out breeding woody plants retain considerable variability [70]. Genetic variation within the species suggests that this species has large effective population size or large mutation rate due to longer generation time. Genetic diversity is essential to the long term survival of tree species. Without it there may be a risk of extinction because of lack of adaptive ability [74].
High genetic diversity would be expected in populations of *C. zeylanicum*, since the taxa is adapted for cross pollination, habitat changes and larger population size in different locations of Karnataka. The overall genetic diversity of a taxon has great implications for its long term survival and continued evolution [12]. Therefore, knowledge of the levels and distribution of genetic diversity is important for designing conservation strategies for threatened and endangered species [113], [52], [132], [133].

5.8 Interspecific genetic diversity study

A clear understanding of the genetic relationships among various species is essential for successful and efficient utilization of the genetic variability present in the related wild species [100], [84], [163], [126]. Interspecific genetic diversity based on RAPD markers is suitable for studying phylogenetic relationships and for authentication of species or varieties. Genetic diversity data is important for designing conservation strategies for threatened and endangered species [71], [72], [50]. Wild species are the rich sources of many wild genes like disease and pest resistant genes.

Present study represents the first step in using RAPD markers as a tool to implement studies of molecular systematics in *Cinnamomum*. The inclusion of other species of *Cinnamomum* and the use of an increased number of primers might provide a greater resolution of the affinities among these species. Also, direct sequencing of amplified bands may be important for recreating phylogenies. The results suggest that RAPD markers are able to differentiate and fingerprint different genotypes that are phenotypically similar.

Diversity analysis is traditionally done based on differences in morphological characteristics, which is influenced mostly by environmental factors. Random amplified polymorphic DNA (RAPD) markers [189] have been successfully used for establishing phylogenetic relationships among plant species. RAPD markers have been extensively employed in establishing phylogenetic relationships in *Cicer* [84], in *Fagopyrum* [163], within *Digitalis* [126], among 9 annual *Cicer* species [2], *Hordeum* [3], *Capsicum* [152], *Chenopodium* [155], *Persea* [48], *Eleucine* [78], etc.
5.9 Genetic relationships among *Cinnamomum* species

RAPD markers represent an efficient and inexpensive tool to generate molecular data and thus have been used successfully in various taxonomic and phylogenetic studies [84], [163], [126]. Out of 40 random decamer primers (operon) used in present study (Table-3), 13 primers were selected on the basis of their robustness of the amplification, reproducibility, clarity and scorability of banding patterns, which were employed for diversity analysis. These primers differed greatly in their efficiency for revealing polymorphism (Table-8).

In the present investigation 8 species of the genus *Cinnamomum* collected from southern part of India were used (Table-2) to assess genetic polymorphism. In all 111 reproducible DNA fragments were amplified, 98\% of which were polymorphic and consistently generated from 13 primers across *Cinnamomum* species. Each primer generated 3 to 16 polymorphic amplification products with an average of 8.5 bands per primer (Table-8). Primer OPB-17 yielded highest number of bands (16), while OPA-19 and OPB-16 amplified the lowest number of bands (3). The approximate size of the largest fragment was 4200 bp whereas the smallest recognizable fragment was 100 bp in size.

RAPD banding pattern was compared to assess genetic variability at the DNA level. Considerable differences in banding pattern were observed. Certain DNA fragments were found to be species specific with some primers and such bands could be used for species identification. Our RAPD survey of 8 *Cinnamomum* species demonstrated that some of the identified RAPD markers were species specific. OPB-17-938 in *C. malabatrum* with primer OPB-17 and OPB-8-1200 in *C.nicolsonianum* with primer OPB-8 were species specific found only in these species and were not found in the remaining species. Such markers may be important for species identification by developing a SCAR-DNA marker and cultivar characterization and can be used to detect instances of natural interspecific gene introgression. Nevertheless, further analysis with more species and primers will be required to establish fully the specificity of loci to particular taxa and subsequent interspecific gene flow in *Cinnamomum*. 
The estimates of pairwise genetic distance between the 8 species of *Cinnamomum* is given in Table-9. The genetic similarity matrix was calculated, based on Jaccard’s similarity indices which ranges from 0.17 between *C.zeylanicum* and *C. travancoricum* to 0.81 between *C.macrocarpum* and *C.travancoricum*. Cluster analysis by using Jaccards similarity coefficient to generate a UPGMA dendrogram shows overall genetic relatedness among the *Cinnamomum* species (Fig-32). Two distinct clusters could be identified, the first cluster comprised wild species of *Cinnamomum* i.e. *C.malabatrum, C.sulphuratum, C.macrocarpum, C.travancoricum, C.wightii, C.nicolsonianum and C.walaiwarense* and the second cluster comprised cultivated *C.zeylanicum*. Species placed within a cluster were more closely related to each other than to species present in different clusters. Thus *C.macrocarpum* and *C.travancoricum* in first cluster are genetically closer to each other as they exhibit high genetic similarity than to other species.

UPGMA cluster analysis evidenced by Principal Component Analysis reveals that *C.macrocarpum* exhibits very close genetic relationship with *C.travancoricum* with 0.81 similarity index. Morphologically, these two species are distinct and *C.macrocarpum* bears the largest fruits among all the Indian *Cinnamomum* species and the crushed leaves are with the smell of aniseed (safrol) and clove (eugenol). Our results of RAPD finger printing could easily distinguish these two species at the molecular level and suggest that both *C.macrocarpum* and *C.travancoricum* can be considered as distinct species. In flora of Karnataka, Saldanha [26] explains that *C.malabatrum* is a synonym of *C.macrocarpum*. However, these two species were treated as independent species by Kostermans [105] in Bulletin of the Botanical Society of India. Our molecular analysis supports the latter as evidenced by less genetic similarity (0.37) between these two species. *C.wightii*, which forms the subcluster, is genetically closer to *C.macrocarpum* and *C.travancoricum* with 0.76 and 0.69 similarity index respectively. *C.sulphuratum* and *C.nicolsonianum* forms separate clusters and exhibit fair genetic relatedness with other species in first cluster. *C.sulphuratum* is more close to *C.travancoricum* with 0.65 similarity index and it is placed almost at the same distance from *C.macrocarpum* and *C.wightii* with 0.60 similarity index. *C.nicolsonianum* exhibits similarity index ranging from 0.54 to 0.25 with *C.macrocarpum* and *C.zeylanicum* respectively suggesting its closeness to *C. macrocarpum* than *C.zeylanicum, C.malabatrum* and *C.walaiwarense* forms a sub
cluster in the first major cluster and are placed at 0.34 similarity index. C.zeylanicum which forms a separate group in second cluster (Fig-32) and shows a poor genetic relationship with other Cinnamomum species in the first cluster with maximum similarity of 0.29 with C.sulphuratum and minimum similarity of 0.17 with C.travancoricum. The Principal Component Analysis (PCA) supports major clustering pattern (Fig-33).

Since most of the Cinnamomum species are used as a source of spice, the presence of a unique RAPD markers among various Cinnamomum species indicates the utility of the approach for fingerprinting purposes. RAPD fingerprinting has a number of potential applications which include the determination of cultivar purity, efficient use and management of genetic resources particularly the establishment of property rights (plant variety protection and patenting). This study represents the preliminary step in using RAPD markers as a tool to develop species specific SCAR-DNA markers for molecular analysis of individual Cinnamomum species.

5.10 SCAR-marker development in C.malabatrum

Analysis of SCAR-SMCM (Scar marker for Cinnamomum malabatrum) through Gene Tool lite version 1.0 revealed that Cinnamomum malabatrum (SMCM) sequence exhibit considerable homology with wheat (Accession Number M 90664.1) ubquitin activating enzyme (E1) mRNA, (Basic Local Alignment SearchTool)(http://www.ncbiblastanalysis/NCBIBlast/Cinnamomummalabatrum.htm and pBLAST [79] ("iHOP - http://www.ihop-net.org/"). Further investigation on this can reveal the significance of this homology with wheat ubquitin activating enzyme, and also it exhibits considerable homology with Vitis vinifera (Accession Number AM 440969).

5.11 Significance and utility of the SCAR markers in species identification and selection for breeding purpose

In south India, a substitute of the real C.tamala leaves was used, most of it is representing C.malabatrum leaves. Spicy leaves and bark of C.malabatrum are substituted or adulterated for the commercial C.zeylanicum bark and leaves used as spice. C.malabatrum leaves have been used as spice and are still sold as an inferior substitute. Bark of C.malabatrum is carminative, antispasmodic, haemostatic,
astringent, antiseptic, stomachic and germicidal. Correct genotype identification of the plant material, therefore, remained important for protection of both the public health and industry. SCAR markers can be used for species identification of genuine *Cinnamomum malabatrum* to distinguish it from common substitutes and adulterants since it is used as alternative spice for *Cinnamomum zeylanicum*. This methodology will be useful in quality control of herbal medicine.

Genotype identification of the plant material remains important for protection of both the public health and industry. Chemoprophiling and morphological evaluation are routinely used for identification of the plants. Chemical complexity and lack of therapeutic markers are some of the limitations associated with chemical approach while subjective bias in morphological evaluation limits its use. Molecular biology offers various techniques that can be applied for plant identification. Development of more specific, sensitive and reproducible markers like RAPD based Sequence Characterized Amplified Region (SCAR) can increase industrial application of the molecular markers. These markers have been used for authentication of medicinal plant species of *Ginseng* [13], *Artemisia* [111], *Phyllanthus emblica* [184], *Pueraria tuberosa* [39], *Embelia ribes* [38], and other commercially important timbers like *Bamboo* [34].

5.12 Conservation considerations

Twenty four *Cinnamomum* species currently redlisted by IUCN, including *C.sulphuratum* and *C.wightii* [170]. Endemic plants of westernghats and other species of *Cinnamomum* are facing great pressure and threat because of economic activities, especially manual picking of bark and fruits of *Cinnamomum* species as spice and for their medicinal value. Due to the unregulated use and overexploitation, medicinal plant number is steadily decreasing. If the necessary conservation measures are not adopted, the species could become extinct. Genetic diversity data are important for conservation and management of rare and endangered species. Maintenance of genetic diversity is essential to the long term survival of the tree species without which there may be a risk of its extinction because of lack of adaptive ability [74]. The study offering practical information for the future conservation and sustainable management programmes for the *Cinnamomum* species are an urgent priority.
6. SUMMARY AND CONCLUSION

1. *Cinnamomum* is an interesting genus with over 250 species. Being the oldest spice plant, *Cinnamomum* exhibits medicinal properties, and many species of *Cinnamomum* yield a volatile oil on distillation. The present study include 8 *Cinnamomum* species out of 13 Indian species found in the western ghat area of Karnataka, Tamilnadu and Kerala.

2. The genetic diversity among 15 natural genotypes of *Cinnamomum zeylanicum* was evaluated by using random amplified polymorphic DNA (RAPD) markers.

3. A Total of 133 different RAPD bands were generated by 11 primers analyzed and pair wise genetic distances were calculated between genotypes according to Jaccard, similarity index and these values were used to construct UPGMA dendrogram.

4. Cluster analysis based on the genetic similarities placed 15 populations into five major groups based on similarity index. High genetic variability was detected within each genotype. 89% of the genetic variation occurred between the genotypes of *C.zeylanicum*, this finding agrees with the behaviour of an out crossing species.

5. PCR based DNA fingerprinting was used to study genetic relationships among 8 species of *Cinnamomum*. Out of the 40 primers initially screened for genetic polymorphism analysis, 13 primers amplified genomic DNA across all the 8 species. A total of 111 amplified bands were observed with an average of 8.5 bands per primer, of which 98% were polymorphic. Pair wise genetic distances were calculated between species according to Jaccard, similarity index and these values were used to construct UPGMA dendrogram.

6. Cluster analysis based on the genetic similarities placed 8 species of *Cinnamomum* into two major clusters with wild species of *Cinnamomum*, i.e., *C. malabatrum, C.sulphuratatum, C.macrocarpum, C.travancoricum, C.wightii, C.nicolsonianum* and *C.walaiwarense* in the first cluster and cultivated *C. zeylanicum* in the second cluster.
7. The results of the present investigation showed that *C. macrocarpum* is very closely related to *C. travancoricum* as evidenced by 0.81 similarity index.

8. The RAPD results distinguished the *C. malabatrum* and *C. macrocarpum* evidenced by 99.63 genetic distance. In flora of Karnataka, Saldanha [26] explains that *C. malabatrum* is a synonym of *C. macrocarpum*. These two species were treated as independent species by Kosterman [105] in the Bulletin of the Botanical Society of India. However, our molecular analysis supports the latter.

9. Classification of species based on morphological characters should be considered after further investigation by using molecular markers.

10. High levels of variability of wild species are expected, because they were not subjected to any of the selection pressures of domestication. Maintenance of high genetic variability would favour their survival under natural condition.

11. Since most of the *Cinnamomum* species are used as a source of spice, the presence of a unique RAPD marker among the various *Cinnamomum* species indicate the utility of the approach for fingerprinting purposes. RAPD fingerprinting has a number of potential applications which include the determination of cultivar purity, efficient use and management of genetic resources, particularly the establishment of property rights (Plant variety protection and patenting). This study represents only the first step in using RAPD markers as a tool to develop species specific SCAR-DNA markers for molecular analysis of individual *Cinnamomum* species.

12. The genetic polymorphism observed among the cultivars is interesting and can be used to develop markers for cultivar identification. Studies comparing the therapeutic efficacy of various cultivars are needed and development of such cultivar specific markers would then be relevant.

13. These results substantiate the applicability of the designed primers as a qualitative diagnostic tool for identification of *C. malabatrum*. However, for quantitative analysis of *C. malabatrum* content in commercial samples, advanced technique such as real time PCR could be tried. Further, there is
pool of material that can be used as adulterant for crude and processed *C. malabatrum* leaves and fruits. The adulterant may be phylogenetically close or distinct from *C. malabatrum*. 
7. LIST OF PUBLICATIONS


