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Full Length Research Paper

Genetic diversity in *Cinnamomum zeylanicum* Blume. (Lauraceae) using random amplified polymorphic DNA (RAPD) markers

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Cinnamomum zeylanicum **Blume. is an important taxa cultivated in coastal plains for its stem bark, leaf, fruits and roots as spice and for its medicinal properties.** ***Cinnamomum*** **is an indicator plant of semi evergreen forest. In this study, RAPD-PCR analysis involving 11 decamer random primers was used to assess the genetic variation within** ***C. zeylanicum*** **in Western Ghats of southern India. Some primers showed appreciable intra-species variation or molecular polymorphism at amplicon levels. Despite morphological similarity, a great deal of genetic polymorphism was observed among the accessions. In this study, unweighed pair group method with arithmetic averages (UPGMA) analysis showed up to 89% genetic variation among these accessions, which is further supported by principle co-ordinate analysis (PCA).**

Key words: Genetic diversity, *Cinnamomum zeylanicum*, RAPD markers, DNA polymorphism.

INTRODUCTION

The genus *Cinnamomum* Schaeffer belongs to the family Lauraceae, which has 32 genera and 2000 to 2500 species. They are mainly evergreen trees of the tropical and subtropical area. *Cinnamomum* is the largest genus in Lauraceae comprising 250 species, distributed in India, Sri Lanka and Australia (Priya and Maridass, 2008). *Cinnamomum* is represented in South India by 12 endemic species and the introduced cultivated species *Cinnamomum verum* (Kostermans, 1983).

The cinnamons of the market are the inner barks obtained from trees of tropical countries and islands. *Cinnamomum zeylanicum* is an ancient, highly prized spice. The genus *Cinnamomum* is of considerable economic importance, cultivated for its bark which is used as a spice, in perfumery and also in ayurvedic and traditional Chinese medicine for its hypoglycaemic, digestive, antispasmodic and antiseptic properties. Bark of the *C. zeylanicum* which is acrid, bitter, sweet and aromatic is reported to be used in treating bronchitis, asthma, nausea, vomiting, flatulence, fever and for restoring normal skin, etc. Cinnamon oil is stomachic, carminative (Sumy et al., 2000). It is reported to be useful in inflamemations, vomiting and tubercular ulcers and diabetes. Cinnamon is mainly employed in the flavouring industry where it is used in meat and fast food seasonings, soft drinks, tobacco flavors, dental and pharmaceutical preparations, cosmetics and perfumes. Bark oil is considered to have a strong germicidal and fungicidal activity. Cinnamaldehyde is anaesthetic, antipyretic, hypotensive, hypothermic and sedative. Oil from number of *Cinnamomum* species has antifungal activity. The major compounds present in both stem and root barks are cinnamaldehyde (75%) and camphor (56%), respectively (Senanayake et al., 1978).

During the last decade, several novel DNA markers (RAPD, RFLP, SSR, ISSR, etc.) have been rapidly integrated into the molecular tools available for genome analysis (Salimath et al., 1995). DNA bands are treated as unit characters and its presence or absence in the amplicon may be used to study genetic relationship (Sang and Soren, 2000) and inter-and intra-specific genetic variations (M' Ribu and Hilu, 1994).

Considering the widespread distribution and marked morphological diversity, including growth habits of plants

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Table 1. *C. zeylanicum* collections, accession numbers and their place of collection.

S/N	Accession number	Collection site in Karnataka, India
1	CZ - 440	Sirsi, Karnataka.
2	CZ - 443	Londa, Karnataka.
3	CZ - 444	Dharwad, Karnataka.
4	CZ - 448	Sonda, Karnataka
5	CZ - 450	Coimbatore, Tamilnadu
6	CZ - 452	Madikeri, Karnataka.
7	CZ - 453	Coimbatore, Tamilnadu
8	CZ - 455	UAS, Dharwad, Karnataka
9	CZ - 456	Yellapur, Karnataka.
10	CZ - 457	Khanapur, Karnataka.
11	CZ - 458	Dandeli, Karnataka.
12	CZ - 460	Sirsi, Karnataka.
13	CZ - 465	Bangalore, GKVK, Karnataka
14	CZ - 475	Bangalore, Karnataka
15	CZ - 478	Silent Valley, Kerala.

within species, it is important to analyze the genetic diversity using molecular tools. PCR-based markers have been used extensively for assessing genetic variation within the species to measure genetic diversity (Virk et al., 1995). In this study, RAPD markers were used to assess the genetic diversity within *C. zeylanicum* populations collected from different geographical locations in the Western Ghats of Karnataka and other states. Frankel (1974) opined that, genetic variation is essential for the long term survival of species and it is a critical feature in conservation. Therefore, tracing successfully adapted variants at genetic level of *C. 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 (Li et al., 2002; Fu et al., 2003; Padmalatha and Prasad, 2006a, b). The objective of this study was to assess genetic diversity among the accessions of *C. zeylanicum* using RAPD markers to provide genetic data and a theoretical basis for protection of the species. Hence, an attempt was made to investigate genetic variation among fifteen accessions of *C. zeylanicum* by using RAPD markers. 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.

MATERIALS AND METHODS

Twenty-five (25) accessions of *C. zeylanicum* were personally collected from different geographical locations in Western Ghats of South India. Fifteen (15) random accessions were used in this study (Table 1). Vouchered specimens are deposited in herbarium, Botany Department, Karnataka Science College, Dharwad and authenticated from the Department of Botany, Bharthiar University Coimbatore, India.

DNA isolation

Genomic DNA was extracted from young leaves following the modified CTAB method described by Doyle and Doyle (1990). Quantification and purity of isolated DNA was checked through uv-spectrophotometer and agarose gel electrophoresis, respectively. The intact double stranded DNA forming a thick single band confirmed the good quality of genomic DNA.

PCR amplification

A set of 40 random primers (Operon Technologies, Almeda, California, USA) were used to amplify the genomic DNA as described by Williams et al. (1990). Amplification reactions were conducted in 20 µl reaction mixture consisting of 100 ng genomic DNA, 5 pM/µl RAPD primer, 10 µM dNTPs, 0.5 µl Taq. DNA polymerase (3U/ µl) (Bagalore Genei, Bangalore, India), 10 X PCR buffer and 1.5 mM $MgCl_2$. DNA amplification was carried out using a gradient palm cycler (Corbett Research, Australia) with the following programme, 1 cycle at 94°C for 4 min for initial denaturation, followed by 40 cycles each of 94°C for 15 s (denaturation), 35°C for 15 s (primer annealing), 72°C for 1.15 min (extension) and final extension at 72°C for 7 min and 4°C for 3 min. The RAPD products were analyzed on 1.5% (w/v) agarose gels in 1X TBE under constant voltage of 100 v for 3 to 4 h. Only best two gel photographs are given in Figure 1. Experiments were repeated several times to ensure the reproducibility and the best gels of the replicates were used for band scoring. The gels were stained with ethidium bromide solution and documented in a gel documentation system. A Phix 174 DNA/ Hae III digest and 1 kb ladder (Banglore Genei, India) were used as molecular size markers.

Data analysis

Each polymorphic DNA band was considered to be a unit character

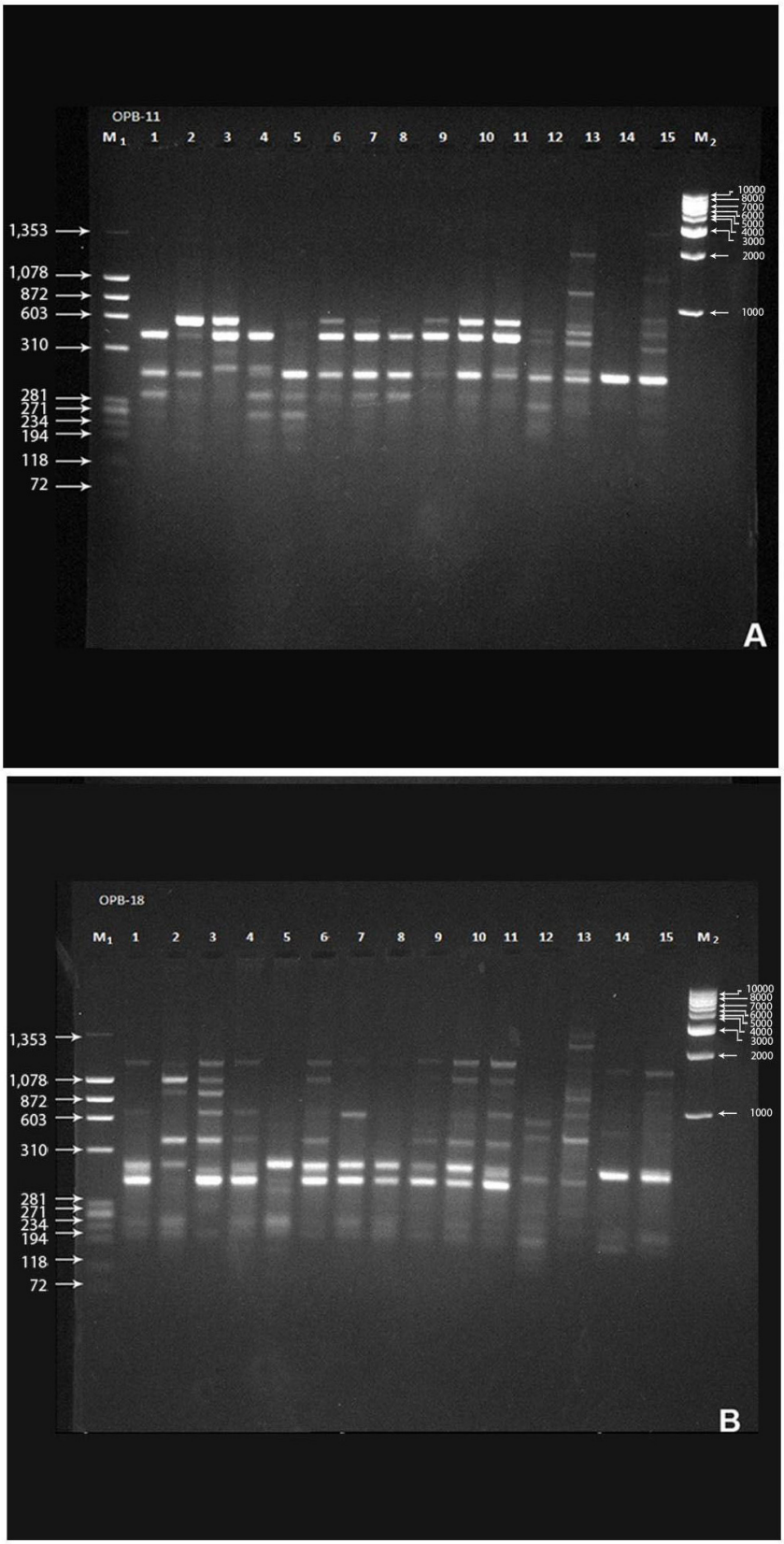

Figure 1. RAPD profiles of 15 accessions of *C. zeylanicum* using primers A, OPB-11 and B, OPB-18. (M_1): Phix/Hae III, (M_2): 1 kb ladder and 1 to 15, *C. zeylanicum* accessions.

Table 2. Details of randomly selected decamer oligonucleotide primers.

S/N	Primer	Nucleotide sequence 5' ⟶ 3'	Number of amplified band	Number of polymorphic band
1	OPB-07	GGTGACGCAG	15	12
2	OPB-08	GTCCACACGC	11	11
3	OPB-10	CTGCTGGGAC	10	8
4	OPB-11	GTAGACCCGT	9	7
5	OPB-13	TTCCCCCGCT	12	12
6	OPB-16	TTTGCCCGGA	10	10
7	OPB-17	AGGGAACGAG	16	15
8	OPB-18	CCACAGCAGT	14	11
9	OPB-19	ACCCCCGAAG	13	12
10	OPB-20	GGACCCTTAC	14	13
11	OPA- 04	AATCGGGCTG	9	7

and the populations were manually scored as binary data with the presence as "1" and absence as "0". Only clearly distinguishable DNA bands were used in the genetic analysis. The molecular size of the amplified products was calculated from a standard curve based on the known size of the DNA fragments of the ladder. Estimates of genetic similarity were calculated between all pairs of the accessions according to similarity index (Jaccard, 1908). The matrix obtained was used to evaluate the genetic relationship among accessions of *C. zeylanicum* with cluster analysis using an unweighed pair group method with arithmetic averages (UPGMA) (Sneath and Sokal, 1973). All the statistical analysis were performed with an aid of NTSYS-PC computer program version 2.0, version 16.0 (Rohlf, 1997). PCA analysis was performed to indicate the genetic relationship among the accessions of *C. zeylanicum* which are geographically isolated.

RESULTS

Fifteen (15) accessions of *C. zeylanicum* revealed 89% of polymorphism with 11 RAPD primers. A total of 133 bands were scored of which 119 bands are polymorphic. The number of bands ranging from 9 to 16 per primer corresponds to an average of 12 bands (Table 2). Jaccard's similarity matrix reveals that, the levels of genetic similarity between accessions ranged from 0.33 to 0.87 (Table 3). The maximum number of polymorphic bands (15) were yielded with primer OPB-17 and minimum (7) from OPB-11 and OPA-4 (Figure 1). However, other primers OPB-7, 8, 10, 13, 16, 18, 19, 20 yielded 12, 11, 8, 12, 10, 11, 12, 13 bands, respectively (Table 2).

Dendrogram (Figure 2) reveals the formation of 5 major genetic groups among 15 accessions studied based on the genetic similarity index (Table 3), even though morphologically they were similar and inseparable. The first group is again classified into two sub groups, where the first sub group includes the accessions CZ-440 and CZ-448 while the second subgroup includes CZ-453 and CZ-455. The second group includes 5 accessions and are further classified in to 3 subgroups. First subgroup includes accessions CZ-452 and CZ-456, exhibiting maximum genetic similarity (0.87), second sub group includes single accession CZ-453 and the third subgroup includes accessions CZ-457 and CZ-458. The third group was formed by accessions CZ-443 and CZ-444. The fourth group consists of accessions CZ-475 and CZ-478 and the fifth group includes accessions CZ-460 and CZ-465 (Figure 2).

DISSCUSSION

RAPD markers represent an efficient and inexpensive tool to generate molecular data and thus, have been used successfully in various taxonomic and phylogenetic studies (Iruela et al., 2002; Sharma and Jana, 2002; Nebauer et al., 2000; Jayaram and Prasad, 2008).

High level of genetic similarity is expected among *C. zeylanicum* accessions restricted to Karnataka due to similar geographical conditions. But in contrast, this study reveals broad genetic base indicating earlier introduction of this species and subsequently, leading to accumulation of variation.

The genetic differentiation of accessions of *C. zeylanicum* could broadly be explained as a result of abiotic (geographical for example, hydrographic connections or climatic differentiation) and biotic (pollination between populations and seed dispersal) factors. The percentage polymorphism (89%) among *C. zeylanicum* is comparatively much higher than other endangered plants, for example, *Cathaya argyrophylla* (32%) (Wang et al., 1996), *Paeonia rockii* (33.3%) (Su et al., 1999), a vulnerable medicinal plant *Oroxylum indicum* (49.61%) (Jayaram and prasad, 2008), *Costus speciosus* (35%) (Asit et al., 2007); this clearly indicates high level of genetic diversity within *C. zeylanicum*.

High level of genetic diversity in populations of *C. zeylanicum* may be due to cross pollination by insects, dispersal of seeds, habitat changes and larger population size in different locations. This is a consequence of

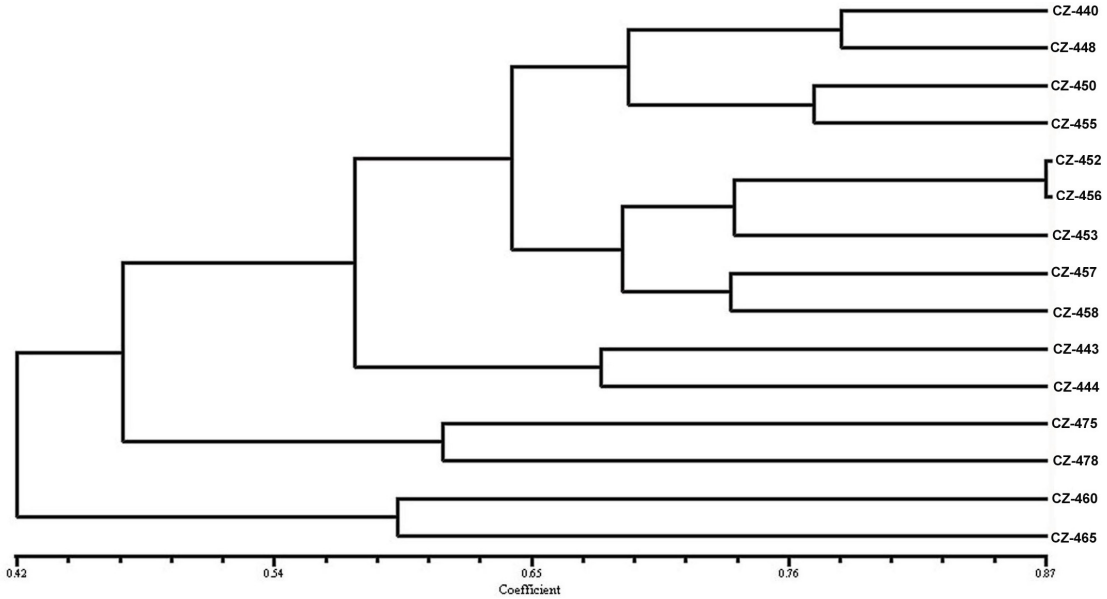

Figure 2. Dendrogram showing genetic diversity of *C. zeylanicum* accessions based on RAPD data.

Table 3. Similarity matrix of 15 accessions of *C. zeylanicum* based on RAPD data.

	CZ-440	CZ-443	CZ-444	CZ-448	CZ-450	CZ-452	CZ-453	CZ-455	CZ-456	CZ-457	CZ-458	CZ-460	CZ.465	CZ-475	CZ-478
CZ-440	1.00														
CZ-443	0.55	1.00													
CZ-444	0.52	0.68	1.00												
CZ-448	0.78	0.60	0.56	1.00											
CZ-450	0.65	0.54	0.40	0.64	1.00										
CZ-452	0.72	0.66	0.63	0.63	0.63	1.00									
CZ-453	0.77	0.60	0.52	0.70	0.71	0.75	1.00								
CZ-455	0.78	0.5	0.41	0.70	0.77	0.66	0.75	1.00							
CZ-456	0.67	0.66	0.61	0.63	0.57	0.87	0.73	0.66	1.00						
CZ-457	0.61	0.61	0.55	0.58	0.63	0.70	0.64	0.62	0.70	1.00					
CZ-458	0.64	0.65	0.72	0.65	0.48	0.71	0.64	0.51	0.74	0.74	1.00				
CZ-460	0.45	0.47	0.49	0.5	0.46	0.51	0.52	0.47	0.51	0.42	0.44	1.00			
CZ.465	0.33	0.38	0.49	0.36	0.34	0.41	0.37	0.32	0.38	0.39	0.43	0.59	1.00		
CZ-475	0.45	0.39	0.37	0.42	0.54	0.47	0.48	0.60	0.53	0.5	0.41	0.42	0.35	1.00	
CZ-478	0.45	0.45	0.45	0.4	0.52	0.47	0.45	0.5	0.46	0.55	0.51	0.38	0.43	0.61	1.00

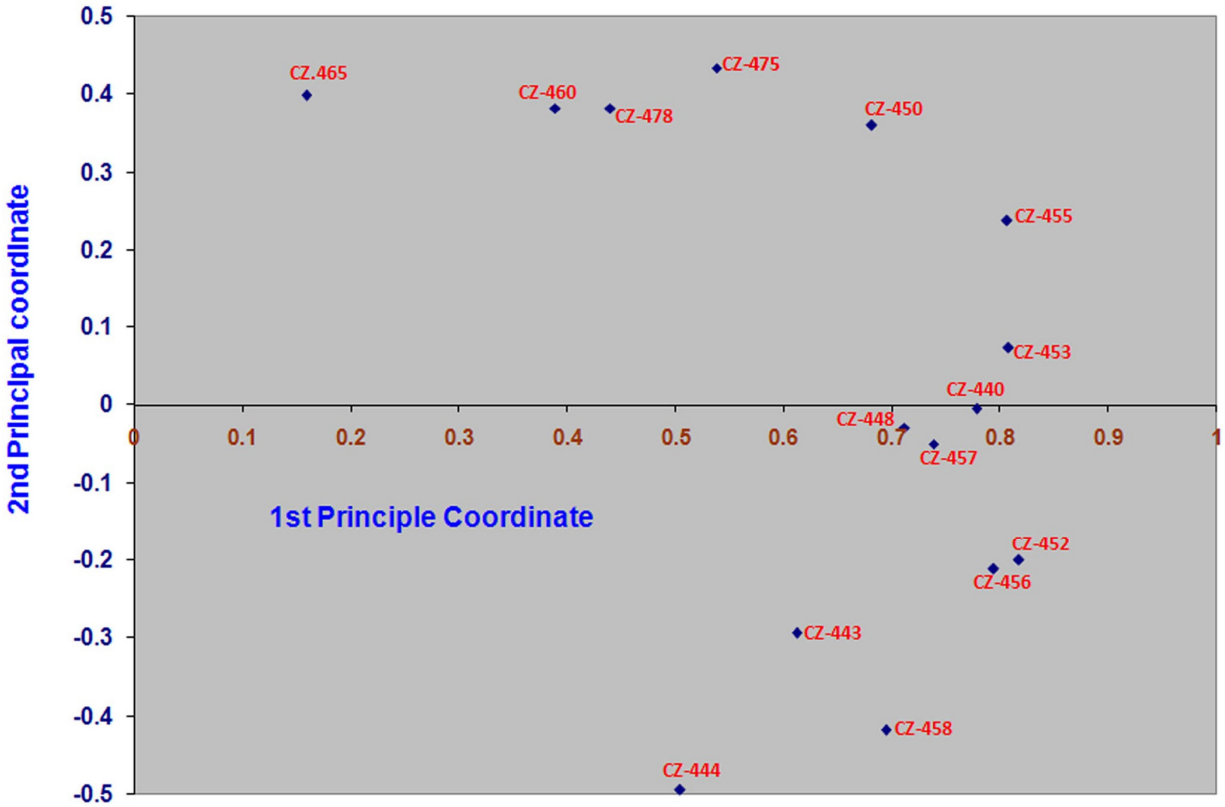

Figure 3. Principle co-ordinate analysis (PCA) of 15 accessions of *C. zeylanicum*.

important gene flow (long distant dispersal seed by mammal) and the characteristics of old domesticated species. The significant degree of variation (similarity index 0.33) between accessions CZ-440 and CZ456 reveals maximum genetic diversity as a result of geographical isolation and change in the environmental conditions. There is a close genetic similarity of 87% observed between accessions CZ-456 and CZ-452 which clearly depict that, genetically the similarity may be because of the similar environmental conditions. The principle coordinate analysis supports the major clustering pattern (Figure 3). In essence, the RAPD method used in this study displayed appreciable intra-population variation or molecular polymorphism, which is pre-existed in different collections. In spite of their morphological similarity, substantial polymorphism was observed among the accessions under study. 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 accessions collected from 15 locations. The dendrogram also established genetic relatedness among different accessions and quantum of changes that occurred in the genome in the course of evolution. This study confirms the suitability of RAPD as a reliable, simple, easy to handle and elegant tool in molecular diagnosis of different accessions of *C. zeylanicum* available in Western Ghats of Karnataka. Currently, it is also proved that, the entries that were found to be similar in taxonomical classification based on morphological characteristics do have divergence at DNA level. Vast genetic variation is an indicative of the evolving nature of the taxa.

Conclusion

The study was undertaken to evaluate the extent and range of genetic diversity available in *C. zeylanicum*. Accurate estimates of diversity are a prerequisite for optimizing sampling strategies and for conserving tree genetic resources. The high diversity revealed by RAPD is in agreement with the conclusion that, out breeding woody plants retains considerable variability (Hamrick, 1990). Variation within the species suggests that, this species has large effective population size or large mutation rate due to longer generation.

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