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RESEARCH ARTICLE

Preliminary investigation for the identification of Sri Lankan *Cinnamomum* species using randomly amplified polymorphic DNA (RAPD) and sequence related amplified polymorphic (SRAP) markers

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Abstract: Cinnamon is one of the important minor export crops in Sri Lanka. *Cinnamomum verum* Presl is known as true cinnamon and seven other wild *Cinnamomum* spp. such as *C. dubium* Nees, *C. citriodorum* Thw., *C. capparu–coronde* Blume, *C. litseaefolium* Thw., *C. ovalifolium* Weight*,* *C. rivulorum* Kostermans and *C. sinharajaense* Kostermans are native to Sri Lanka. The identification of *Cinnamomum* species is still based on morphological characters, which are influenced by environmental factors. Therefore, molecular markers such as randomly amplified polymorphic DNA (RAPD) and sequence related amplified polymorphism (SRAP) were employed in order to find a more reliable approach to identify *Cinnamomum* species correctly. Using RAPD and SRAP techniques, it was possible to detect the polymorphism as well as to identify the *Cinnamomum* species. Fourteen RAPD primers and 20 sets of SRAP primer combinations gave amplification products. However, one set of SRAP primer combinations produced more markers for *Cinnamomum* species than the RAPD markers. Amplification products from both techniques could be categorized as genus specific, species specific and intra-species specific markers. Both polymerase chain reaction (PCR) based techniques employed in this study can be used to identify the species, to estimate the genetic diversity of *Cinnamomum* spp. and to detect polymorphism, which could be used to screen the accessions of germplasm collected at the Department of Export Agriculture, Sri Lanka.

Keywords: *Cinnamomum* spp.*,* cinnamon, RAPD, SRAP, Sri Lanka.

INTRODUCTION

The genus *Cinnamomum* belongs to Family Lauraceae. It consists of about 250 species distributed in the South, East and South East Asia and Australia (Kostermans, 1995). *Cinnamomum verum* Presl known as true cinnamon is one of the important spice crops in Sri Lanka. In addition to *C. verum* Presl, the genus includes several other important wild cinnamon species such as *C. capparu-coronde, C. citriodorum*, *C. dubium, C. litseafolium, C. ovalifolium*, *C. rivulorum* and *C. sinharajanse* (Sritharan, 1984), reported to be present in Sri Lanka. Currently, the extent of cinnamon cultivation in Sri Lanka is around 29450 ha, which contributes to 60 − 70 % of the total world production (Anon, 2006). Although *C. verum* is the main commercial species, other species of *Cinnamomum* are reported to have medicinal and ethno-botanical values (Krishnamoorthy *et al*., 1997; Khan *et al*., 2003; Bruno, 2009; Kumarathilake *et al*., 2010; Smerq & Sharma, 2011).

Morphological characters (leaf and floral characters) are used to identify *Cinnamomum* species in Sri Lanka, particularly for field identification. Morphological features such as leaf length (L), breadth (B), area, petiole length, apex angle (A^o), base angle (B^o) and mean B^o/A^o are useful in characterizing the *Cinnamomum* species. Among the wild species, leaf length, breadth and petiole length was the highest in *C. sinharajanse* and lowest in *C. ovalifolium* (Sritharan, 1984). The mean L/B ratio was found to be a useful parameter to separate the species from one another, e.g. *C. capparu-coronde* and *C. rivulorum* had values > 3, while *C. ovalifolium* and unidentified individuals had values between 1 and 2 and the rest

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had values between 2 and 3 (Sritharan, 1984). Some of these species are significantly different from each other and can be easily distinguished by examining the leaf morphological characters, e.g. *C. dubium* has densely grey-tomentellous flush, midrib and two prominent basal lateral veins running out below the acumen to almost the tip of leaf. The flush of *C. liseaefolium* is silvery-sericeous, the midrib of the leaves are faint and the two basal or sub-basal veins terminate at the middle of leaf. Leaf shapes are quite different even within a species, e.g. leaf shape of *C. verum,* can vary from oval or elliptical to lanceolate-oval or narrowly elliptical (Dassanayaka *et al*., 1995).

Many studies of these species are based on the identification of floral and pollen morphology, ecophysiological features, chemical and essential oil constituents etc. (Joshep, 1981; Wijesinghe *et al*., 2004; Dighe *et al*., 2005; Saumyasiri *et al*., 2006). Among the *Cinnamomum* species the largest size of pollen was recorded from *C. verum* (26.23 ± 0.24 µm) and the smallest (17.62 ± 0.14 µm) from *C. dubium* (Sritharan, 1984). Based on the stomatal indices, *Cinnamomum* species can be categorized into 3 groups, those with the lowest stomatal index: 18 − 19 (C. *sinharajanse*, *C. revulorum* and *C. capparu coronde*), the highest stomatal index: 25 − 27 (*C. ovalifolium* and *C. liseaefolium*), and medium stomatal index: 22 (*C. verum* and *C. dubium*).

Considering the above facts, the identification of *Cinnamomum* species using only morphological and floral characters has difficulties and may lead to controversy. Although there are morphological variations among *Cinnamomum* spp., the morphological data alone may not be sufficient to examine the relationships within and between the species. Since environmental factors and environmental plasticity may greatly influence the morphology of plants, reliability of the morphological data is very low (Russell *et al*., 1993).

In this context, molecular characterization of the plant species would be quite helpful to distinguish the species in terms of taxonomy (Sharma *et al*., 2008). Numerous molecular techniques have been developed in recent years for visualizing DNA sequence polymorphism. There are a large number of molecular markers; random amplified polymorphic DNA–RAPD (Williams *et al*., 1990), amplified fragment length polymorphism–AFLP (Vos *et al*., 1995), simple sequence repeat polymorphism (SSR) and other polymerase chain reaction (PCR) marker systems (Karp *et al*., 1998). Molecular markers have been used to characterize numerous plants (Karp *et al*., 1998). RAPD has shown to be a very useful technique for species identification (Abeysinghe *et al*., 1999; 2000), construction of genetic maps (Tingey & Del Tufo, 1993), DNA fingerprinting (Williams *et al*., 1990; Karp *et al*., 1998), identification of genetic polymorphism and the parentage determination (Welsh *et al*., 1991). Sequence-related amplified polymorphism (SRAP) markers have been used in taxonomy (Ai *et al*., 2011), genetic diversity analysis in grasses (Budak *et al*., 2004), breeding (Wu *et al*., 2011), genetic map construction and gene tagging in *Brassica oleracea* (Li & Quiros, 2001) and gene tagging and cloning in *B. rapa* (Zhang *et al*., 2009). Molecular markers have been used not only to resolve the fragmentary status of the taxonomy of plant species such as mangroves (Abeysinghe *et al*., 1999) and other plant species (Bandelj *et al*., 2002; Arif *et al*., 2010), but also to distinguish species for breeding purposes and to facilitate conservation (Bardakci, 2001).

Studies have been undertaken to investigate the genetic variation and verify the taxonomic status of the *Cinnamomum* species at molecular level (Lin *et al*., 1997; Kojoma *et al*., 2002; Soulange *et al*., 2007; Joy & Maridass, 2008; Kuo *et al*., 2010; Lee *et al*., 2010; Ho & Hung, 2011; Sandigawad & Patil, 2011; Kameyama, 2012). However, a very few studies have been conducted to study the cinnamon phylogeny in Sri Lanka. Saumyasiri *et al*. (2006) have studied the phenetic relationships of wild and cultivated accessions of cinnamon in Sri Lanka.

Abeysinghe *et al*. (2009) have carried out research on the genetic analysis of *Cinnamomum* species by sequencing *Trn*L intron region, intergenic spacers between *trn*T-*trn*L, *trn*L-*trn*F*, trn*H -psbA and nuclear ITS. This is the only record of a molecular level study of *Cinnamomum* species in Sri Lanka. There is a germplasm collection of 700 accessions of *C. verum* from all parts of Sri Lanka maintained at the Cinnamon Research Station, Department of Export Agriculture, Palolpitiya, Thihagoda, Matara, based mainly on some morphological and agronomic characters. It is possible that there may be an undetermined number of duplicates in the collection. Therefore, the aim of this research was to use molecular marker systems to check whether they could be used to study the taxonomy of cinnamon species found in Sri Lanka and to examine whether there is intra-specific variation, which will be useful to screen the *C. verum* accessions of the germplasm for breeding purposes.

METHODS AND MATERIALS

Collection of plant materials

From the available *Cinnamomum* species at the Cinnamon Research Station (CRS) young leaf samples were obtained from two individuals of *C. dubium*, *C. capparu-coronde*

Blume, *C. citriodorum*, *C. litseaefolium*, three individuals of *C. rivulorum* and four individuals of *C. verum* and four *C. verum* selected accessions (CRS 357, CRS 23, CRS 184 and CRS 318). As a preliminary study, samples were collected only from the CRS. At the time of collection, young suitable leaf material was not available for *C. sinharajaense* and *C. ovalifolium*. The collected leaf materials were brought to the laboratory of the Department of Botany, University of Ruhuna, Sri Lanka, preserved using silica gel and stored until the DNA was extracted.

DNA extraction and amplification for RAPD

The DNA was isolated from young silica dried leaf tissues of *Cinnamomum* species using the hexadecetyl–trimethylammonium bromide (CTAB) procedure (Doyle & Doyle, 1990) with some modifications such as the use of 3 % CTAB and addition of 2 % PVP– polyvynylpyrylidone. Quality and the quantity of extracted DNA were checked on agarose gel. They were non degraded and there was sufficient amount of DNA for PCR. This DNA was used for preliminary RAPD and SRAP analysis. The amplification of genomic DNA was done using 20 RAPD primers from Operon Technology (Alamenda, California, USA). Since there were no molecular data available for Sri Lankan *Cinnamomum* species for either RAPD or SRAP, primers for PCR were used randomly. The details of each primer are shown in Table 1. PCR was performed according to the protocol of Williams *et al*. (1990). Initially 20 primers (OPA-07, OPA-15, OPE-01, OPE-07, OPE-09, OPE 14, OPE-15, OPE-16, OPG-14, OPG-15, OPH-11, OPI-01, OPJ-04, OPJ-11, OPJ-12, OPO-06, OPT-06, OPT-12, OPT-15 and OPT-16) (Table 1) were screened using PCR to check whether these primers have the ability to amplify and give reproducible DNA fragments. Two replicates were performed for each PCR. The fragments generated by amplification were resolved on 1.5 % agarose gel and observed under UV illuminator.

PCR amplification for SRAP and fragment analysis

Twenty six different SRAP primer combinations were used with three different labelled primers: Em1 (red coloured), FC1 (blue coloured) and SA7 (green coloured). With SA7 labelled primer, 8 different unlabelled primers (BG1, BG5, Bg48, BG56, BG62, BG68, BG78 and BG93), with FC1, 16 unlabeled primers (BG1, BG11, BG14, BG15, BG22, BG25, BG48, BG60, BG62, BG66, BG67, BG68, BG73, BG84, BG85, BG93) and with Em1, 2 unlabelled primers (Bg10 and Bg13) were used for the amplification using DNA from 5 *Cinnamomum* species (4 individuals of *C. verum*, 2 individuals of *C. capparu-coronde*, *C. citriodorum* and *C. dubium* and 3 individuals of *C. rivulorum*). PCR was conducted in a final volume of 10 µL and the PCR conditions were as follows; initially denaturing template DNA at 95 ˚C for 3 mins followed by the first 5 cycles, which consisted of 50 s at 95 ˚C for template denaturation, 50 s at 35 ˚C for primer annealing and 1 min at 72 ˚C for primer extension. This was followed by 30 cycles; each cycle consisted of 50 s at 95 ˚C for template denaturation, 45 s at 50 ˚C for primer annealing and 1 min at 72 ˚C for primer extension and one cycle of 10 min for the completion of primer extension. The amplified products were run in an ABI 3100 automated sequencer and analyzed for amplified fragments with different lengths. These different size fragments were analyzed using the Genographer software.

RESULTS

Primer screening

In order to screen and select the suitable RAPD primers, DNA from *C. dubium* and *C. capparu-coronde* was used for PCR. Size of the fragments obtained for primers were in the range of ~300 – ~500 base pair (bp). In this study, fourteen out of the twenty RAPD primers

Table 1: RAPD primers, primer sequences and size of the amplified products

Primer	Primer sequence 5′→3′	Size of the fragment	Primer	Primer sequence 5′→3′	Size of the fragment
OPA-07	GAAACGGGTG	>450 bp	OPH-11	CTTCCGCAGT	Not amplified
OPA-15	TTCCGAACCC	Not amplified	OPI-01	ACCTGGACAC	Not amplified
OPE-01	CCCAAGGTCC	>300 bp	OPJ-04	CCGAACACGG	>400 bp
OPE-07	AGATGCAGCC	>350 bp	OPJ-11	ACTCCTGCGA	>350 bp
OPE-09	CTTCACCCGA	Not amplified	OPJ-12	GTCCCGTGGT	>450 bp
OPE-14	ACTGGGACTC	>400 bp	OPO-06	CCACGGGAAG	Not amplified
OPE-15	ACGCACAACC	>350 bp	OPT-06	CAAGGGCAGA	>300 bp
OPE-16	GGTGACTGTG	>500 bp	OPT-12	GGGTGTGTAG	>350 bp
OPG 14	GGATGAGACC	>450 bp	OPT-15	GGATGCCACT	>450 bp
OPG-15	ACTGGGACTC	Not amplified	OPT-16	GGTGAACGCT	>300 bp

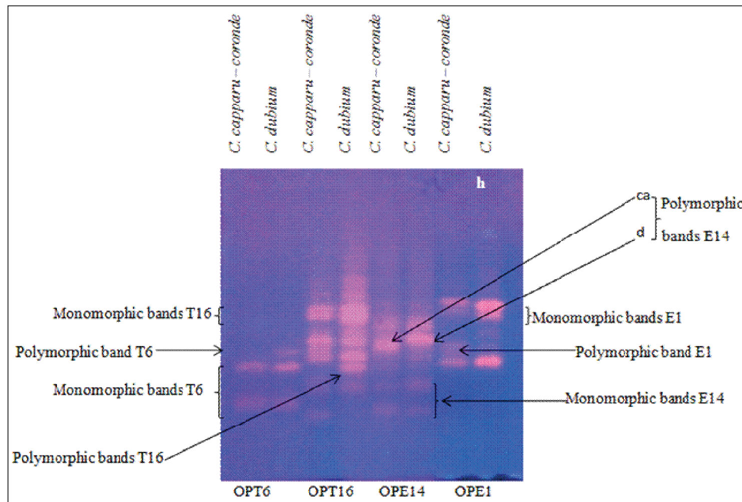

Figure 1: Preliminary screening of RAPD primers: OPT6, OPT16, OPE14 and OPE1 using DNA from *C. capparu-coronde* (ca) and *C. dubium* (d)

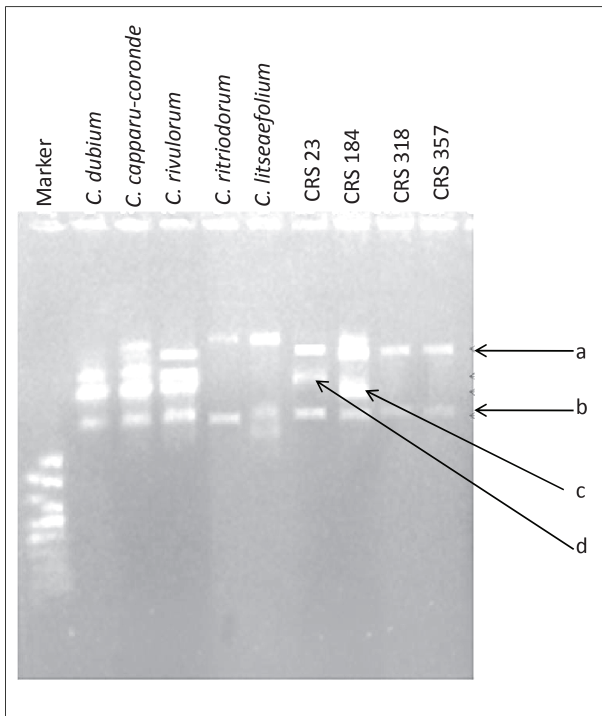

Figure 2: Variation with the RAPD primer OPA-7 of *C. dubium*, *C. capparu-coronde*, *C. rivulorum*, *C. citriodorum*, *C. litseaefolium* and *C. verum* accessions: CRS 23, CRS 184, CRS 318, and CRS 357. Species specific marker a for *C. verum* and generic specific marker b for all species. c and d markers could be considered as polymorphic bands for *C. verum.*

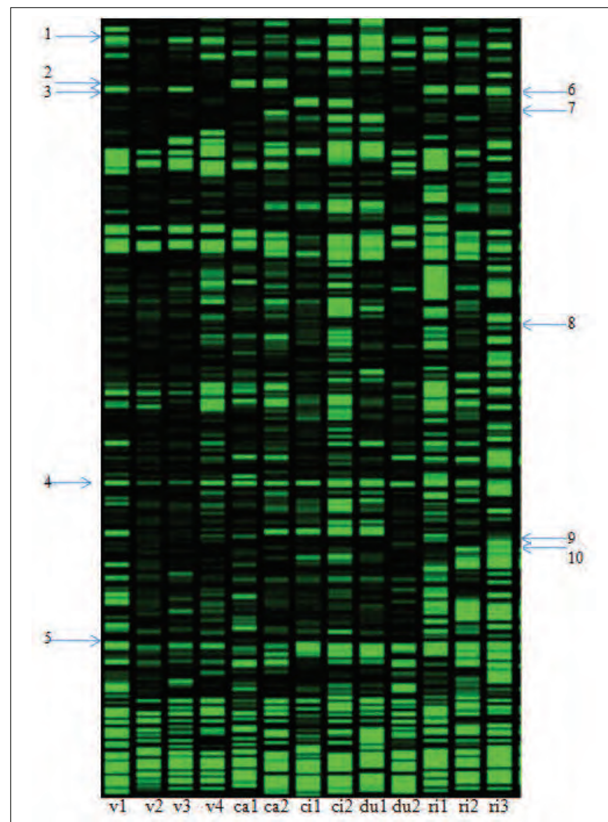

Figure 3: SRAPs amplified by green coloured labelled SA7 and with unlabelled bg48 primers with cinnamon species: v1-v3 *C. verum* individuals, ca1 and ca2 *C. capparu-coronde* individuals, ci1 and ci2 *C. citriodorum* individuals, du1 and du2 *C. dubium* individuals, ri1 and ri2 *C. rivulorum* individuals. Generic, species or polymorphic markers, 1 − 10 are shown with arrows. Markers present and absent in *Cinnamomum* species are tabulated in Table 2.

tested (Table 1) were able to amplify the genomic DNA and produce scorable bands (polymorphic bands and monomorphic bands) in the tested species. The RAPD patterns were analyzed for polymorphism (Figure 1). The 14 primers namely, OPA-07, OPE-01, OPE-07, OPE 14, OPE-15, OPE-16, OPG-14, OPJ-04, OPJ-11, OPJ-12, OPT-06, OPT-12, OPT-15 and OPT-16 gave amplification products while the others did not give any amplification. The number of fragments generated per primer varied between 2 – 9. OPE 14, OPG 14, OPT 06, OPT 15 and OPT 16 primers gave a higher number of polymorphic bands compared to others. The average number of amplification products for species ranged from 2 – 9. However, some primers gave highly polymorphic banding patterns, which were more than five scorable bands compared to others primers.

RAPD analysis

The banding patterns obtained from the preliminary screening with primers, OPT-06, OPT16, OPE14 and OPE-01 is shown in Figure 1. With OPT-06 primer, there is one polymorphic band (T6), which is present in *C. dubium* but absent in *C. capparu-coronde.* Therefore, this band could be considered as a species specific marker for *C. dubium*. The monomorphic band T6 could be considered as the genus specific marker since it is present in both species. Likewise polymorphic bands T16 and E14d can be considered as the species specific markers for *C. dubium* and polymorphic bands E14ca E1 can be considered as the species specific markers for *C. capparu-coronde*, while monomorphic bands T16 and E14 can be considered as the genus specific markers for both species. According to the preliminary study, monomorphic bands (genus specific) and polymorphic RAPD bands (species specific) were observed. Considering the RAPD products of the primer OPA-07 (Figure 2), species specific marker 'a' for *C. verum* and genus specific marker 'b' for all species could be identified. Moreover, polymorphism among the individuals (c and d markers) of the species *C. verum* could be observed.

Table 2: SRAP markers originated from SA7 and with unlabelled bg48 primers

Marker	v1	v2	v3	v4	ca1	ca2	ci1	ci2	du1	du2	ri1	ri2	ri3	Remarks
1	+	+	+	+	+	+	+	+	+	+	+	+	+	Generic specific marker
2	0	0	0	0	+	+	0	0	0	0	0	0	0	Species specific marker
3	+	0	+	0	0	0	0	0	0	0	0	0	0	Polymorphic marker for *C. verum*
4	+	+	+	+	+	+	+	+	+	+	+	+	+	Generic specific marker
5	+	+	+	+	+	+	+	+	+	+	+	+	+	Generic specific marker
6	0	0	0	0	0	0	0	0	0	0	+	+	+	Species specific marker for *C. rivulorum*
7	0	0	0	0	0	0	+	+	0	0	0	0	0	Species specific marker for *C. citriodorum*
8	0	0	0	0	0	0	0	0	0	0	+	0	+	Polymorphic marker for *C. rivulorum*
9	0	0	0	0	0	0	0	0	0	0	0	0	+	Polymorphic marker for *C. rivulorum*
10	0	0	0	0	0	0	0	0	0	0	0	+	+	Polymorphic marker for *C. rivulorum*

v1-v3 *C. verum* individuals, ca1 and ca2 *C. capparu-coronde* individuals, ci1 and ci2 *C. citriodorum* individuals, du1 and du2 *C. dubium* individuals, ri1 and ri2 *C. rivulorum* individuals

\+ indicates the presence of a fragment and 0 indicates the absence of a marker.

SRAP markers for *Cinnamomum*

Out of the 26 SRAP primer combinations 4 primer combinations gave the polymorphic banding pattern. These primer combinations produced genus specific, species specific and polymorphic markers for *Cinnamomum* species (Figure 3 and Table 2). According to Figure 3, SARP has produced more markers per one combination than RAPD. Using this marker system, it was possible to identify the *Cinnamomum* species and study the genetic diversity. Genus specific, species specific and polymorphic markers or intra-species markers for each species could also be observed (Figure 3 and Table 2). Table 2 shows the genus specific markers (fragments 1, 4 and 5), species specific marker for *C. capparu-coronde* (fragment 2– ca1 and ca2), species specific marker for *C. rivulorum* (fragment 6– ri1, ,ri2 and ri3), species

specific marker for *C. citriodorum* (fragment 7– ci1 and ci2), and polymorphic marker for *C. rivulorum* (fragments 8, 9 and 10).

DISCUSSION

The foremost objective of this research was to study the variation of *Cinnamomum* species at DNA level. By employing different oligonucleotide primers (RAPD primers), molecular markers can be generated that are diagnostic at different taxanomic levels. For any given primer, RAPD amplification products can be broadly classified into two groups: variable (polymorphic) or constant (non-polymorphic). These definitions are relative for a given taxanomic unit (OTU) (Hadrys *et al*., 1992). Similar kinds of RAPD products were observed in this study. Although RAPD has some disadvantages such as co-migration of different size fragments and reproducibility problems, RAPD markers have been applied in cultivar identification (Cabrita *et al.,* 2001), parentage determination (Elisiàrio *et al*., 1999) and identification of inter-specific hybrids (Lee *et al*., 2006; Saitou *et al*., 2007).

The RAPD analysis of individuals within the species *C. verum* (CRS23, CRS184, CRS318 and CRS357) and six species within the genus (*C. verum, C. capparu-coronde*, *C. citriodorum, C. dubium,* and *C. rivulorum*) has identified constant fragments diagnostic for a genus. Therefore, these genus specific fragments can serve as diagnostic DNA markers in RAPD analyses. The species specific fragment is identified by its presence in all the individuals of *C. verum* (CRS23, CRS184, CRS318 and CRS357). By employing fragments that are polymorphic among the individuals, RAPD analysis could be used to assess the diversity of *Cinnamomum* species.

Genetic markers with RAPD are obtained for the identification of polymorphism based upon the existence of polymorphic RAPD bands. Therefore, it is important to identify as many RAPD bands as possible. High degree of polymorphism observed indicates a potential for selection and availability as a genetic source (Bandelj *et al*., 2002). Therefore, RAPD primers OPE-14, OPG-14, OPT-06, OPT-15, and OPT-16 can be used for further studies to detect polymorphism in *Cinnamomum* spp. It is however essential to carry out more research using all species with more samples, as well as with replicates (at least two) for each primer to get more polymorphic bands (variation) in order to conclude the delimitation of *Cinnamomum* spp. This study was carried out as a preliminary primer screening for the available leaf samples of *Cinnamomum* species during the collection of samples for DNA extraction. Therefore, further research should be carried out to study the variation of *Cinnamomum* species with expanded sampling of individuals of each species in order to get a better picture of the species relationship.

Another PCR based technique, SRAP was used to check whether this technique can be utilized to identify the *Cinnamomum* species, and to study the diversity of *Cinnamomum* species found in Sri Lanka. In this study five *Cinnamomum* species (*C. verum, C. capparu-coronde*, *C. citriodorum, C. dubium* and *C. rivulorum*) found in Sri Lanka was investigated using SRAP markers. Out of the 26 SRAP primer combinations 4 primer combinations amplified the DNA producing large number of markers per one primer combination. This preliminary study showed that using these molecular markers, it is possible to identify the *Cinnamomum* species (genus specific and species specific) and intra-species variations. Since both molecular marker systems gave genus specific and species specific markers, these markers could be used to identify the *Cinnamomum* species correctly. Both RAPD and SRAP markers gave intra-specific polymorphic banding patterns among the individuals of *C. verum*, and this marker system could be used to screen the 700 accessions present at the Cinnamon Research Station, Department of Export Agriculture, Palolpitiya, Thihagoda, Matara, Sri Lanka to examine their genetic relatedness. However, before the screening of the germplasm, it is necessary to select more reliable and consistent RAPD and SRAP markers.

The results generated by molecular studies are in agreement with previous morphology-based analysis (Wijesinghe *et al*., 2004). The existence of intermediate forms blurs the inter-specific and intra-specific relationships within the *Cinnamomum* genus and it has proven difficult to resolve or to distinguish species using only morphological characters. Therefore, this study contributes new molecular data for the identification of different species of *Cinnamomum* that have not been used for molecular marker analysis.

An earlier investigation of the diversity of *Cinnamomum* species (*C. verum*, *C. citriodorum*, *C. capparu-coronde*, *C. dubium*, *C. litseafolium*, *C. rivulorum*, *C. sinharajanse* and *C. camphora*) found in Sri Lanka has been conducted by sequencing the different regions of chloroplast spacer regions of *Trn*L intron region, intergenic spacers between *trn*T-*trn*L, *trn*L-*trn*F and nuclear ITS region of the rDNA (Abeysinghe *et al*., 2009). One mutation at each *trn*L-*trn*F and *trn*T-*trn*L-IGS regions was deteced while four mutation sites were observed in *trn*L intron. The length of the amplified products of *trn*L UAA intron, intergenic spacers (IGS) of

*trn*L-*trn*F and *trn*T-*trn*L were approximately 400 – 600 bp. Since much variations were not observed among the cpDNA regions studied, the two molecular marker systems (RAPD and SRAP) were employed in order to clarify the taxonomy status of cinnamon species found in Sri Lanka.

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