Research Journal of Agriculture and Biological Sciences, 5(6): 1079-1088, 2009
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Molecular Characterization of Cinnamon (*Cinnamomum Verum* Presl) Accessions and Evaluation of Genetic Relatedness of Cinnamon Species in Sri Lanka Based on *Trn*L Intron Region, Intergenic Spacers Between *trn*T-*trn*L, *trn*L-*trn*F, *trn*H -*psb*A and nuclear ITS

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Abstract: *Cinnamomum verum* is one of the most important spice species in Sri Lanka and it contributes to 70% of the world bark production. There are 9 *Cinnamomum* species found in Sri Lanka. Although it is a very important spice crop in Sri Lanka, the taxonomy of the species has a controversy. These species are identified by the floral, foliar morphology and pollen morphology, ecophysiological features, chemical and essential oil constituents and etc. A molecular technique to identify these species would greatly facilitate identification of species correctly. Genetic variation among species of *Cinnamomum*: *C. verum*, *C. citriodorum*, *C. capparu-coronde*, *C. dubium*, *C. litseafolium*, *C. rivulorum*, *C. sinharajaense* and *C. camphora* found in Sri Lanka was studied by analyzing nucleotide sequence of different cpDNA regions and an ITS region of the rDNA. The cpDNA regions studied were intergenic spacers between *trn*L-*trn*F, *trn*T-*trn*L, *trn*H-*psb*A and *trn*L intron. We were able to identify a number of variable sites for cpDNA sequences of *trn*L intron, *trn*L-*trn*F IGS, *trn*T-*trn*L IGS, *trn*H-*psb*A IGS and intergenic transcribed spacer (ITS) regions of rDNA. Although there is not much variations among the cpDNA regions we studied, it will contribute to the clarification of the genetic diversity of Sri Lankan *Cinnamomum* species which have never been studied at the DNA level. Although there is not much cpDNA variation and it did not provide much information for the taxonomy of *Cinnamomum* species in Sri Lanka. But the ITS region of the rDNA was shown to be useful to identify species.

Key words: *Cinnamomum*, cpDNA, *trn*L intron, *trn*L-*trn*F, *trn*T-*trn*L, *trn*H-*psb*A

INTRODUCTION

The genus *Cinnamomum* belongs to the family Lauraceae and consists of about 110 species. The genus includes several economically important species; *C. verum*, *C. cassia*, C. *burmanni*, *C. camphora* etc. *Cinnamomum verum* Presl is known as true Cinnamon. This is one of the most important spices as well as cash crop species in Sri Lanka. The main commercial product of cinnamon trees is the dried bark of the stem in the form of quills, quisllings and chips. The three major parts of the plant: leaf, stem-bark and root-bark yield three different types of essential oils [1,2,3]. Cinnamon is used in the food industry as a spice for flavoring various foods such as pickles, cakes, biscuits, sweets, liquor, in food preservation and in the soap industry. A fat obtained from the fruits was formerly used for candles in churches [4]. Currently, the amount of Cinnamon under cultivation is reported to be around 28,230 ha, which contributes 60-70% of the total world production [5]. Preliminary analysis of floral and morphological characters of *Cinnamomum* in Sri Lanka identified nine species. In addition to true cinnamon, (*Cinnamomum verum* Presl) the other important eight wild species of this genus are *C. citriodorum* Thw., *C. capparu-coronde* Blume, *C. dubium* Nees, *C. litseaefolium* Thw., *C. rivulorum Kosterm*., *C. sinharajaense* Kosterm., *C. ovalifolium* Wight and another unknown *Cinnamomum* species [6] reported to be present in Sri Lanka. There have also been limited phylogenetic studies for *Cinnamomum* found in Sri Lanka. Studies undertaken over the past few decades have dramatically increased the amount of information on the *Cinnamomum* spp. in Sri Lanka. Studies simple have provided greater understanding of foliar morphology, pollen and floral morphology, ecophysiological, features, chemical and essential oil constituents [7]. There is a wide variability of Cinnamon

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in farmer's fields in terms of quantity and quality of stem bark, bark and leaf oil qualities, re-juvenile capacity of plant height, stem diameter and resistance to major pests and diseases etc.

Although there is morphological variation in *Cinnamomum* spp. used in the study of phylogeny, the data is not sufficient to clarify the relationship they have within and between species. Environmental plasticity may influence morphology of the plants to a great extent and hamper the taxonomy. Because of the fragmentary status of the taxonomy of these species, conservation has faced some difficulties. In this context, molecular characterization of these available wild species will be quite helpful to distinguish these species in terms of taxonomy, breeding purposes and facilitate conservation [8,9,10].

To our knowledge no comprehensive studies at the molecular level of the genus, *Cinnamomum* have been undertaken in order to verify the taxonomic status of the *Cinnamomum* species found in Sri Lanka. Since morphological data did not provide strong phylogenetic signal, sequencing of DNA in different regions (trnL intron, *trn*T-*trn*L intergenic, *trn*L-*trn*F intergenic, *trn*H-*psb*A intergenic spacer and rDNA ITS) was undertaken to differentiate these species and to examine the genetic variation among the accessions of *Cinnamomum verum* Presl that have been currently identified in Sri Lanka.

MATERIALS AND METHODS

Sample Collection: Young leaf samples of ten accessions (CRS 23, CRS 40, CRS 83, CRS 156, CRS 166, CRS 184, CRS 201, CRS 317, CRS 318 and CRS 351) of *C. verum* individuals from each *C. citriodorum, C. capparu-coronde, C. dubium, C. litseafolium, C. rivulorum, C. sinharajaense,, C. ovalifolium* and another unknown *Cinnamomum* species were collected (Table 1) from the Cinnamon Research Station, in the Department of Export Agriculture, Palolpitiya, Thihagoda, Matara, Sri Lanka. Then they were labeled, preserved in silica gel and stored until DNA was extracted. A total of 27 individuals representing 9 *Cinnamomum* species were included in this study. *C. camphora was* included as a representative of the outgroup species.

DNA Extraction: Total DNA was isolated from young silica dried leaf tissues from all *Cinnamomum* species using a hexadecetyltrimethylammonium bromide (CTAB) procedure. This procedure is based on Doyle and Doyle [11] with some modifications such as additional washing step, using buffer I and an additional buffer II before addition of the CTAB buffer. Silica dried one cm^2 leaf tissue was placed in a 1.5 mL tube with liquid nitrogen, and was ground to a fine powder. Then, one milliliter of washing buffer I (50 mM Tris–HCl pH 8.0, 0.1% β-mercaptoethanol, 50 mM EDTA, 350 mM sorbitol, 0.1% bovine serum (BSA) albumin, and 10% polyethylene glycol # 6000) was added, and the solution was mixed well, centrifuged 5 minutes at 10,000 rpm and the supernatant was removed. The above washing step was repeated three times using buffer I. Following the third spin, resulting pellet was treated with 300 µL of buffer II (50 mM Tris–HCl pH 8.0, 0.1% β-mercaptoethanol, 5 mM EDTA, 350 mM sorbitol, and 1% sodium sarcosyl) and mixed with 300 µL of 2% CTAB following the procedure of Doyle and Doyle (1990). Concentration and quality of the extracted DNAs were checked by 0.8% agarose gel electrophoresis with λ *Hind*III size marker.

Primers used in PCR: To examine the level of sequence variation of ten accessions of *Cinnamomum verum* Presl and eight different controversial species, the chloroplast *trn*L (Intergenic sequence) UAA intron and adjacent *trn*L-*trn*F IGS, *trn*T-*trn*L IGS universal primers c and d, e and f and a and b respectively, designed by Taberlet *et al.*, [12] were used for amplification of DNA. The *trn*H-*psb*A spacer was amplified using the primers *trn*H-*psb*A developed by Sang *et al.*, [13]. ITS 4 and ITS 5A primers were used to amplify the ITS region described by Stanford *el al.*, [14].

PCR Amplification and Sequencing of DNA:PCR was conducted in a final volume of 20 µL in 0.2 mL PCR tubes in an ASTEC Thermal cycler. The 20 µL of PCR mixture contained 2 µL of *Ex Taq* 10X reaction buffer, 2 mM $MgCl_2$, 200 µM of each forward and reverse primers, 0.2 mM concentration of each DNTP, 0.2 U of *Takara Ex Taq* DNA Polymerase (Takara Bio Inc.) and 50-100 ng of template DNA. PCR was carried out by initially denaturing template DNA at 95 ^{0}C for three minutes followed by 30 cycles. Each cycle consisted of 45 seconds at 95^{0}C for template denaturation, 45 seconds at 50^{0}C for primer annealing and one minute at 72^{0}C for primer extension and one cycle of ten minutes for completing the primer extension.

After checking whether the required fragment was successfully amplified or not by gel electrophoresis of PCR products on 0.8 % agarose gel, PCR products were purified using an enzymatic a cleaning method for sequencing with PCR product cleaning kit (Roche Applied Science). Two sequencing reactions were performed for each purified PCR sample using each primer in 0.2 mL PCR tubes. Sequencing reaction mixture consisted of 160 µM primers, 2 ng template DNA obtained from purified PCR product, 20 µL of BigDye and 20 µL of BigDye buffer, sterile double distilled water to make up a final volume of 10 µL. The purified PCR products were sequenced using the

BigDye Termintor Cycle Sequencing Ready Reaction kit (Applied Biosystems), with the primers used for PCR. This mixture was subjected to initial denaturing at 96^{0}C for two minutes. This was followed by 25 cycles of 20 seconds at 96^{0}C for template denaturation, 10 seconds at 50^0 C for primer annealing and four minutes at 60^{0}C for primer extension. Reactions from the BigDye Terminator Cycle Sequencing Ready Reactions were cleaned to remove unincorporated dye terminators by precipitating them with 1 µL of 3 M sodium acetate and 100 µL of 100% ethanol for 15 minutes at room temperature. The samples were then spun at 10000 rpm for 15 minutes. The supernatant was removed and the samples were washed with 70% ethanol. The samples were air dried and kept at -20^{0}C until used. Prior to running the samples in an ABI 3100 automated sequencer, the samples were resuspended in 15 µL of Hi-Di Formamide (Applied Biosystems). Both strands of the labeled DNA samples were run on an ABI 3100 automated sequencer using capillary electrophoresis and were directly sequenced for all taxa. Whenever possible sequences were generated more than one individual to check whether there is any intra-specific heterogeneity. Sequences were edited and complementary strands were assembled using SeqMan software (DNASTAR). For each sequence, base pair positions were checked for agreement with those of the complementary strands.

Sequence Analysis: A data matrix for sequences of each cpDNA region was constructed using Molecular Evolutionary Genetics Analysis (MEGA4). All sequences were initially aligned using ClustalW implemented in Molecular Evolutionary Genetics Analysis (MEGA4), and then the alignment was visually adjusted to correct any obvious misalignments. All the overlapping sequences had been checked, consensus sequence for all individuals was generated, aligned and the final data matrix was produced. cpDNA aligned sequenced data of all the regions analyzed were combined together and used in the final phylogenetic analyses. Phylogenetic analyses were run with MEGA software version 4.0. The level of support for branches of the NJ tree was determined using the Bootstrap Neighbor-Joining (NJ) analyses using MEGA4. In addition to the analysis of the combined dataset, the following data partitions were analyzed; intron sequence alone (introm matrix), and intron and spacer sequences combined (intron + spacer matrix).

Cloning: PCR products obtained from ITS primers of some individuals showed double peaks or lacked clear base reading. PCR products were first cleaned using QIAGEN MiniElute PCR purification kit following manufactures instructions. Cleaned PCR product was inserted into pGEM-T easy vector and transformed into *E. coli* JM109 in order to amplify respective ITS copies separately. The transformation mixture was incubated in LB medium at 37^{0}C with agitation and plated on LB agar with ampicillin (50 µg/mL), IPTG, and X-Gal (25 µg). Eight white colonies per cloning reaction of each individual were randomly chosen for PCR reaction and the fragments were amplified using M13 forward and reverse primers. In order to minimize the enzymatic error during the amplification, Go Taq DNA polymerase (Promega) was used since this polymerase has proof reading activity.

Phylogenetic Analysis: To infer, phylogenetic relationships among species, rapidly evolving non-coding regions; *trn*L intron, *trn*L-*trn*F IGS, *trn*T-*trn*L IGS, *trn*H-*psb*A IGS and ITS were used. The data marix of the *trn*L intron, *trn*L-*trn*F, cp *trn*T-*trn*L and *psb*A-*trn*H integenic spacers were analyzed by Neighbor-Joining method using MEGA4. Parsimony analyses were also performed using heuristic searching with 500 runs. In the Neighbor-joining method, we used the k-2-p distance and ignored gaps.

RESULTS AND DISCUSSION

cpDNA Variation: There is a germplasm collection of (*Cinnamomum verum* Presl at the Cinnamon Research Station, in the Department of Export Agriculture, Palolpitiya, Thihagoda, Matara, Sri lanka consisting of about 700 accessions, which has been established mainly based on some morphological and agronomic characters. Species identification of *Cinnamomum* species has been done using foliar and floral morphological characters, anatomical features, pollen structure, chemical constituents etc. A scoring system based on these characters resulted in identification of ten superior accessions (CRS 23, CRS 40, CRS 83, CRS 156, CRS 166, CRS 184, CRS 201, CRS 317, CRS 318 and CRS 351) out of these seven hundred accessions [7]. However, these methods are not reliable due to certain problems with the methodology. In addition to true cinnamon (*Cinnamomum verum* Presl), the genus includes the other important eight wild species (Table 1): *Cinnamomum, C. capparu*-coronde, *C. citriodorum*, *C. dubium, C. litseafolium, C. rivulorum, C. sinharajaense, C ovalifolium* and another unknown *Cinnamomum* species [6] reported to grow in Sri Lanka. These are not considered as commercial species. Some of these species are so different from others and can only be distinguished as different species by examining morphological characters. However, the existences of intermediate forms blur

Table 1: Species of *Cinnamomum* and number of accessions and individuals in each species used in this analysis.

Species	No. of individuals
Cinnamomum verum Presl accessions	10 (CRS 23, CRS 40, CRS 83, CRS 156, CRS 156a, CRS 156b, CRS 166, CRS 184, CRS 201, CRS 317, CRS 318 and CRS 351)
C. citriodorum	2
C. capparu-coronde	2
C. dubium	4
C. litseafolium	3
C. rivulorum	2
C. sinharajaense	2
C. camphora	1
Unknown *Cinnamomum* species	1

the interspecific relationships within the *Cinnamomum* and it has been proven difficult to resolve or to distinguish species using only morphological characters. Thus we investigated the *Cinnamomum* species found in Sri Lanka using sequence analysis of different regions of the cpDNA to discriminate the species. To our knowledge this is the first report of the genetic diversity at the DNA level in *Cinnamomum* spp. found in Sri Lanka.

In phylogenetic studies, molecular data of chloroplast (cp) and single copy nuclear genes provide valuable information (Taberlet *et al*.,)[12,15]. Although there are different types of data for plant molecular phylogenetic analyses, cpDNA sequence data is being widely used in the form of a restriction site or DNA sequence analyses [16]. The use of cpDNA in phylogenetic analysis in plant species has been discussed [17]. Since there are multiple copies per chloroplast, a relatively high copy number of chloroplast genome(s) can be obtained very easily within a typical genomic DNA extraction. The properties of cpDNA genome such as structural stability, haploid, uniparental inheritance and high copy number together with a large number of sequence data available in the gene banks facilitate PCR amplification of specific cpDNA regions and thus systematic studies [12]. Also nuclear rDNA has been used in many diversity studies[16,17,18].

Size of the Amplified Products: The primers used in this study allowed amplification of nearly the complete sequence of each intron or IGS (Intergenic Sequence) region in all individuals. In this study we used the *trn*L intron, *trn*L-*trn*F IGS, *trn*T-*trn*L IGS, *trn*H-*psb*A IGS of chloroplast genome and intergenic transcribed spacer (ITS) region of the nuclear genome that are often used in phylogenetic studies for DNA sequencing. The primers used in this study successfully amplified much of the sequence between the primers of each intron or intergenic spacer (IGS) region in all individuals. The length of the sequences obtained for *trn*L intron, *trn*L-*trn*F IGS, *trn*T-*trn*L IGS, *Trn*T-L IGS *trn*H-*psb*A IGS and intergenic transcribed spacer (ITS) were 396-527, 294-360, 367-556, 335-457 and 680-750 bp respectively. We obtained partial sequences for *trn*L-*trn*F IGS, *trn*T-*trn*L IGS, *trn*H-*psb*A IGS and ITS2 for *Cinnamomum* spp. The genus, including a large number of species, out of which eight are found in Sri Lanka, were studied for the first time using DNA sequence analysis.

cpDNA Sequence Variation Within an Individual: Amplification products of 4 different regions of the cpDNA from 9 different *Cinnamomum* species were evaluated. The number of variable sites for sequences of the *trn*L intron, *trn*L-*trn*F IGS, *trn*H-*psb*A IGS and intergenic transcribed spacer (ITS) regions were 4, 1, 1, 9 and 7 respectively (Fig. 1, Fig. 2 and Fig. 3). Although total numbers of polymorphic sites were 15 for cpDNA, all polymorphic sites were not phylogenetically informative. *C. camphora* was found to be more diverged from the other *Cinnamomum* species. There were 4 polymorphic sites found in the *trn*L intron region for *C. camphora.* Among the accessions, it was monomorphic except the CRS156 accession; while variation was observed among other species (Fig. 1). *C. camphora* which was used as an out-group in this study differed at four sites in the *trn*L intron region. All accessions of *C. verum* except CRS 156 and *C. sinharajaense* had the same sequence while *C. citriodorum* and *C. camphora* had a similar type of sequence. *C verum* accession CRS156, *C. dubium*, *C. rivulorum*, the *Cinnamomum* unknown species had a mutation at 130 of the sequence. Kojoma *et al*., [19] found three polymorphic sites in the *trn*L intron. All were polymorphic and phylogenetically informative. The sequences of *trn*L-*trn*F IGS and *trn*T-*trn*L IGS are so conservative that only 2 variable sites were found among the 9 species under study. In the *trn*L-*trn*F IGS

	trnL intron				*trn* L-F	*trn* T-L intergenic
	130	267	278	314	51	364
CRS23	G	C	C	G	T	T
CRS40	.	.	.	.	.	.
CRS83	.	.	.	.	.	.
CRS156	A	.	.	.	C	.
CRS166	.	.	.	.	.	A
CRS184	.	.	.	.	.	.
CRS201	.	.	.	.	.	.
CRS317	.	.	.	.	.	.
CRS318	.	.	.	.	.	.
CRS351	.	.	.	.	.	.
C. capparu coronde	.	.	.	.	.	.
C. citriodorum	A	T	.	T	C	.
C. dubium	A	.	.	.	C	A
C. litseaefolirum	0	0	0	0	.	.
C. rivulorum	A	.	.	.	C	A
C. sinharajaense	.	.	.	.	.	.
Cinnamomum unknown	A	.	.	.	C	A
C. camphora	A	.	T	T	C	.

Fig. 1: Sequence variation of *trn*L intron sequences of *C. verum* accessions, CRS 23, CRS 40, CRS 83, CRS 156, CRS 166, CRS 184, CRS 201, CRS 317, CRS 318 and CRS 351 and other species of *Cinnamomum*. Variations of the sequence were observed at 130, 267, 278 and 314 of the sequences. Variation of the sequences for *trn*L-F and *trn*T-L intergenic sequences were observed at 51 and 364 of the sequence. 0 indicates the not used in this study

region, Kojoma *et al.*, [19] found one mutation site. Our results are comparable with the results of 16 Kojoma *et al.*, [19]. The sequence of *trn*T-*trn*L IGS is so conservative that it did not show much sequence variation among the 9 species under study. Variation was observed at 364 of the nucleotide sequences of *C. verum* accession, 166, *C. dubium* Nees keep, *C. rivulorum* and *Cinnamomum* unknown species. In contrast, nine *Cinnamomum* cpDNA polymorphic sites were found in the *trn*H-*psb*A IGS region (Fig. 2), among which 5 were polymorphic in the Sri Lankan *Cinnamomum* species. These variants were observed among only four *Cinnamomum* species, two accessions of *C. verum, C. citriodorum,* unknown species and *C. litseafolium*. Five different types of sequences were observed in the Sri Lankan *Cinnamomum* species.

Visual alignments of the sequences were achieved easily indicating extreme conservation of the sequences of all regions of DNA under study. According to the results obtained from the three intergenic and one inton of plastid DNA sequences, all cpDNA under study were highly conservative and the number of insertions and deletions were limited. Because the cpDNA data sets had little sequence variation, all cpDNA data sets were combined in order to get concatenated sequence. After the alignment, the data set was used for phylogenetic analysis. The first 50-60 bp of each fragment was discarded since there were ambiguities in those sequences obtained from all individuals. Therefore, the complete aligned sequences were 1155 bp long.

	22	49	109	131	137	138	153	159	185
CRS23	A	C	C	G	A	G	A	C	T
CRS40	.	.	.	.	.	.	.	.	.
CRS83	.	.	.	.	.	.	.	.	.
CRS156	.	.	.	.	.	.	.	.	.
RS166	.	.	.	.	.	.	T	.	.
CRS184	.	.	.	.	.	.	.	.	.
CRS201	.	.	.	.	.	.	T	.	.
CRS317	.	.	.	.	.	.	.	.	.
CRS318	.	.	.	.	.	.	.	.	.
CRS351	.	.	.	.	.	.	.	.	.
C. capparu coronde	.	.	.	.	.	.	.	.	.
C. capparu coronde 1	.	.	.	.	.	.	.	.	.
C. citriodorum	C	.	.	.	.	.	.	.	.
C. citriodorum	C	T	.	.	.	.	.	.	.
C. citriodorum 2	C	T	.	.	.	.	.	.	.
C. dubium	.	.	.	.	.	.	.	.	.
C. dubium 1	.	.	.	.	.	.	.	.	.
C. dubium 2	.	.	.	.	.	.	.	.	.
C. itseaefolirum	.	.	.	T	.	.	.	.	.
C. itseaefolirum 1	.	.	.	T	.	.	.	.	.
C. itseaefolirum 2	.	.	.	T	.	.	.	.	.
C. itseaefolirum 3	.	.	.	.	.	.	.	.	.
C. rivulorum	.	.	.	.	.	.	.	.	.
C. rivulorum 1	.	.	.	.	.	.	.	.	.
C. sinharajaense	.	.	.	.	.	.	.	.	.
C. sinharajaense 1	.	.	.	.	.	.	.	.	.
C . unknown	.	.	.	.	.	.	.	T	.
C. camphora	C	.	T	.	T	T	.	.	C

Fig. 2: Sequence variation of *psb*A-*trn*H intergenic sequences of *C.* verum accessions and other species of *Cinnamomum*. The higher variation, 9 variable sites were observed and the sequences of *psb*A and *trn*H intergenic sequences at 22, 49, 109, 131, 137, 138, 153, 159 and 185 the sequences.

C. camphora was separated from the other Sri Lankan *Cinnamomum* species in the NJ tree indicating the separation from the other species studied (Fig. 4). Even though a slightly higher rate of evolution of cpDNA compared to that of mtDNA was noted, some non-coding regions of cpDNA often fail to provide significant phylogenetic information at lower taxonomic levels [19]. These observations are in agreement with our phylogenetic analysis. Separate analyses of the cpDNA regions resulted in unresolved trees due to a small number of informative characters contained in any one of the four regions.

Fig. 4. shows that cpDNAs of 30 individuals representing 9 species of *Cinnamomum* scattered among all parts of the tree. *C. citriodorum* individuals formed a separate cluster. However, the BT value for this clade was 62% and not so high. The topology of the obtained phylogenetic tree was not consistent with that of the individuals based on morphological data. In the cpDNA tree, members of several morphologically defined taxa were scattered among several cluseters with moderate bootstrap support (64%- 93%).

ITS and Morphological Variation: In order to compensate this limitation as well as to obtain on additional and independent estimate of the phylogeny of *Cinnamomum* species, a second marker system, ITS, was used to improve inferences of the phylogenetic tree. Analyses of the highly evolving ITS region have

	106	199	258	433	481	536	621
CRS40 a	G	G	C	A	T	C	G
CRS40 b	.	.	.	.	.	.	.
CRS40 c	.	.	.	.	.	.	.
CRS40 d	.	.	.	.	.	.	.
CRS40 h	.	.	.	.	.	.	.
CRS166 c	A	A	.	G	.	.	.
CRS166 b	.	A	.	G	.	.	.
CRS156 c	.	A	.	G	.	.	.
CRS23 a	.	.	.	.	.	T	.
CRS23 b	.	.	.	.	.	T	.
CRS23 g	.	.	.	.	.	T	.
CRS83 a	.	.	.	G	.	.	.
CRS83 b	.	.	.	G	.	.	.
CRS83 c	.	.	.	G	.	.	.
CRS83 d	.	.	.	G	.	.	.
CRS201 a	.	.	.	G	C	.	.
CRS201 b	.	A	.	G	.	.	.
CRS201 e	.	A	.	G	.	.	.
CRS201 f	.	A	.	G	C	.	.
CRS201 h	.	A	.	G	.	.	.
C. litseafolium a	.	A	A	G	.	.	A
C. litseafolium b	.	A	A	G	.	.	A
C. litseafolium c	.	A	A	G	.	.	A
C. rivilorum e	.	.	A	G	.	.	.
C. rivilorum f	.	.	A	G	.	.	.
C. rivilorum g	.	.	A	G	.	.	.
C. sinharagensis	.	A	A	G	.	.	.
C. sinharagensis '	.	A	A	G	.	.	.

Fig. 3: Sequencing of the cloned PCR products of ITS regions of the nuclear genome showed 7 variable sites of the studied species at 106, 199, 258, 433, 481, 536 and 621 of the sequences. Alignment of the ITS sequences revealed considerable sequence polymorphism among the different individuals in the same species of all species under study.

been extensively used in many flowering plants [18]. After the alignment, excluding gaps, the total aligned ITS region was 668 bp in length with seven polymorphic sites. All were phylogenetically informative. ITS exhibited high level of sequence variation in *Cinnamomum* species. The phylogeny generated by the ITS sequence data is in agreement with the previous morphology-based analysis (Fig. 5).

C. verum accessions, *C. rivulorum* and *C. sinharajaense, C. litseafolium* were used for the ITS sequence analysis. Sequences of clones of PCR products from different species showed different types of sequences. Clones of the *C. verum* accessions had seven different types of rDNA sequences and they were diverged. *C. litseafolium*, *C. rivulorum* and *C. sinharajanse* respectively had different types of sequences but within the species the sequences were the same (Fig. 5). According to the cluster analysis of the ITS sequences, it is clear that C. *litseafolium*, *C. rivulorum* and *C. sinharajaense* are separated well while the accessions of *Cinnamomum verum* appeared on different branches and not clearly separated according to accessions (Fig. 5). Because we could not obtain sequences of the out-group, we could not determine the root of the tree. However, if the root is located at the point indicated by the arrow in Fig. 5, all *C.* verum accessions were separated from the other *Cinnamomum* species.

In contrast to cpDNA, the ITS region was quite variable between species as well as between *C.* verum accessions. The NJ tree based on ITS sequences was more phylogenetically informative than all chloroplast sequences under investigation. Accessions and species were separated into eight groups. In the NJ tree, the clades of CRS23 and CRS40 were separated with 65% and 61% BT respectively. All the *Cinnamomum* verum accessions are grown in the same field and *Cinnamomum* spp. are known to be cross pollinated.

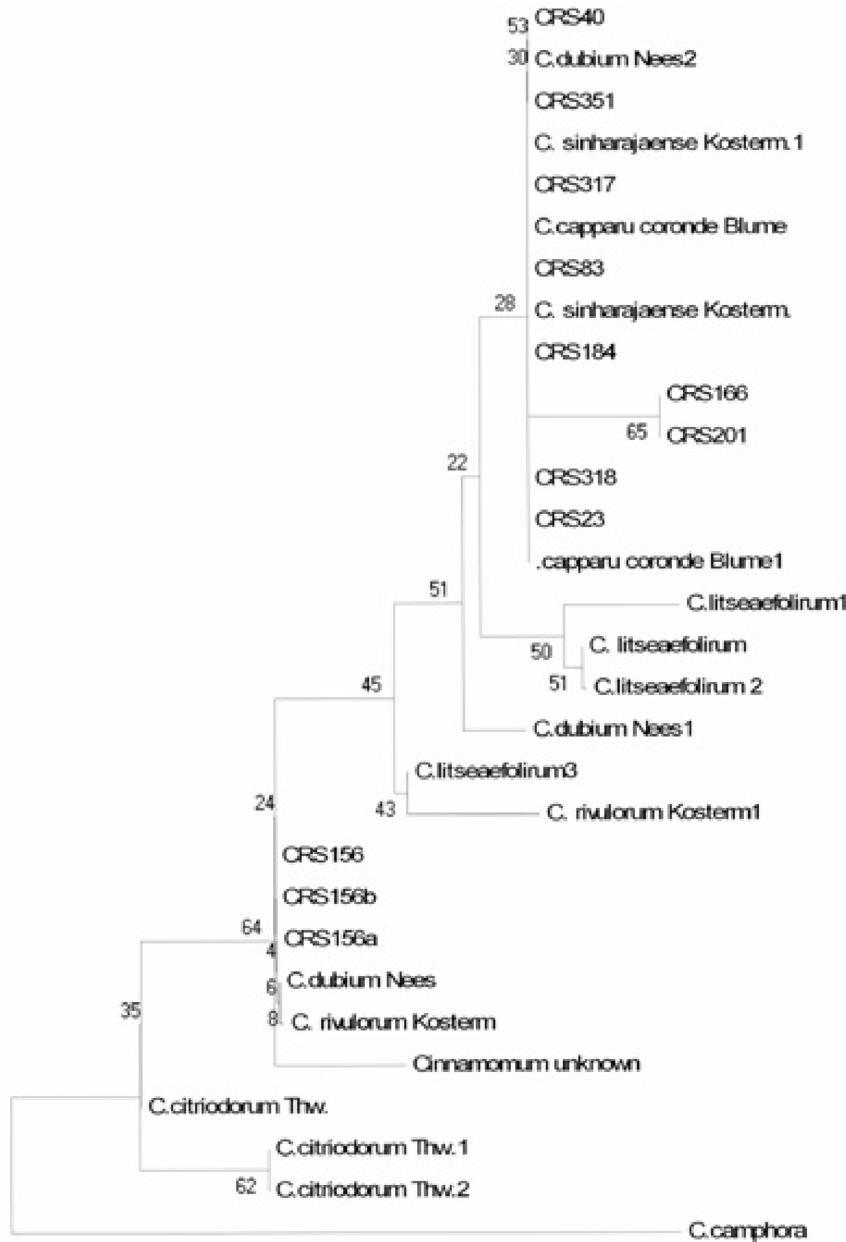

Fig. 4: Phylogenetic relationships of species found in Sri Lanka based on all cpDNA sequences.

Therefore, these individuals seem to be sympatric, hybridize and produce a fertile hybrid in nature. *C. litseafolium*, *C. sinharajanse* and *C. rivulorum* formed separate clades with supports of 65%, 41% and 52% BT values, respectively (Fig. 5). In contrast to the cpDNA topology, the topology of the ITS tree was highly compatible with the present taxonomy of *Cinnamomum* species found in Sri Lanka.

Conclusions: A phylogenetic relationship between species based on cpDNA remains unclear. However, sequences of the ITS region varied between species as well as between *C.* verum accessions. Eight different types of ITS sequences were found within the *Cinnamomum verum* species. Also *C. litseafolium*, *C. rivulorum* and *C. sinharajaense* had different sequences with no variation within species. This study provides new sequencing data for the individuals from different species of *Cinnamomum* that have never been used for sequence analysis. Therefore, it is worthwhile to study nuclear gene variation of *Cinnamomum* species with expanded sampling of individuals in order to get a better picture of the species relationship.

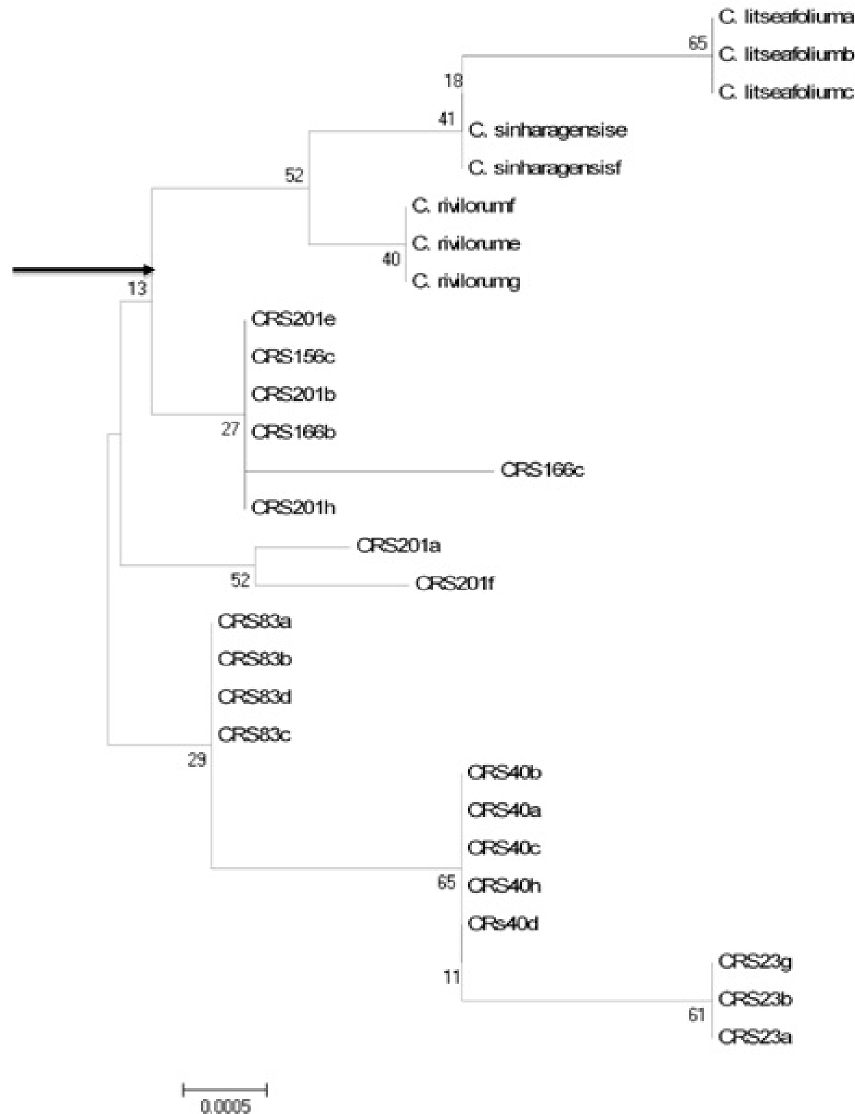

Fig. 5: Cladogram based on ITS data. Numbers represents the bootstrap values.

ACKNOWLEDGMENT

This work was partly funded by the National Science Foundation (NSF) under the Overseas Special Training Programme (OSTP), Sri Lanka.

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