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Possibility of utilizing Inter Simple Sequence Repeat regions, bark powder morphology and floral morphometry to characterize the *Cinnamomum* species in Sri Lanka

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ABSTRACT

The genus *Cinnamomum* of the family Lauraceae is an economically important crop. The Ceylon cinnamon (*Cinnamomum verum syn. C. zeylanicum*) has an exceptional position in the global cinnamon market. In addition to the cultivated species, Sri Lanka is home to seven endemic wild Cinnamomum species, *C. capparu-coronde, C. citriodorum, C. dubium, C. litseifolium, C. ovalifolium, C. rivulorum,* and *C. sinharajaense. Nevertheless,* the species delimitation has not been successful with some morphometric and molecular traits. Therefore, this study focused on molecular characterization with an Inter Simple Sequence Repeats (ISSR) nuclear marker, floral morphometric characteristics, and microscopy of the powered bark of *Cinnamomum* species reported in Sri Lanka. According to the results four polymorphic ISSR regions resulted in an average of 83.7% polymorphism among all collected species, suggesting considerable polymorphism. The bark fiber size of the cultivated species is different from the studied wild species and could be used as a key to identify adulterants during export. The cultivated cinnamon, *C. verum* and two wild species *(C. sinharajaense,* and *C. capparu-coronde)* have considerably larger flowers compared to other species. Moreover, floral traits such as flower colour and shape could differentiate species *C. sinharajaense* and *C. capparu-coronde*. The Scanning Electron Microscopy (SCM) of pollen showed that the pollen size, spine length, interspinal distance, and spine ornamentation significantly contribute to species variation, and such variations need to be studied comprehensively. Nevertheless, the phylogeny of *Cinnamomum* species could not be completely resolved using ISSR regions, bark powder morphology, and floral morphological traits assessed and suggested for further studies.

INTRODUCTION

The Genus *Cinnamomum* consists of over 250 species distributed worldwide (Kostermans, 1957; 1980). Of them, *Cinnamomum verum* J. Presl (syn. *Cinnamomum zeylanicum* Blume), *Cinnamomum cassia* (L.) J.Presl (syn. *Cinnamomum aromaticum* Nees), *Cinnamomum tamala* (Buch.-Ham.) T.Nees (Barceloux, 2009) & C.H.Eberm., *Cinnamomum burmannii* and *Cinnamomum loureiroi* (Chen *et al*., 2014) *Cinnamomum pauciflorum* Nees and *Cinnamomum camphora* (L.) J.Presl got an economic value (Bottoni *et al*., 2021) and are traded as raw materials or value-added products. Sri Lanka shares the highest portion of true cinnamon, also known as Ceylon cinnamon, contributing to about 90% of the world's production in 2020 (Senaratne *et al*., 2020).

In addition to the cultivated species, Sri Lanka's rain forests occupy seven endemic species of *Cinnamomum*; *Cinnamomum capparu-coronde* Blume, *Cinnamomum citriodorum* Thwaites, *Cinnamomum dubium* Nees., *Cinnamomum litseifolium* Thwaites, *Cinnamomum ovalifolium* Wight., *Cinnamomum rivulorum* Kosterm, and *Cinnamomum sinharajaense* Kosterm (Dassanayake *et al*., 1980). Among them, *C. sinharajaense and C. rivulorum* are restricted to Sinharaja rain forests (Kumarathilake *et al*., 2011). *Cinnamomum citriodorum* (R. de Kok, 2021a) and *C. rivulorum* (R. de Kok, 2021c) are categorized as endangered, while other wild species in the vulnerable category (R. de Kok, 2021b; A. De Kok, 2021) except *C. dubium,* included as near threatened species (R. de Kok *et al*., 2020). H*owever, C. dubium, C. capparu-coronde,* and *C. ovalifolium* grow in several agro-ecological zones (Kumarathilake *et al*., 2011).

Recent preliminary work suggests the presence of medicinally important chemicals in several wild species (Liyanage, Madhujith, and Wijesinghe, 2017; Prathibhani *et al.*, 2021). However, sustainable utilization and conservation of these precious species are challenging because of the unavailability of proper identification methods. There is limited literature on morphology-based identification methods (Bandusekara *et al*., 2020; Prathibhani *et al*., 2021). Sritharan and colleagues studied pollen variation among wild species using light microscopy (Sritharan *et al*., 1992). A recent SCM study included two wild species, *C. dubium* and *C. litseifolium* found in Sri Lanka (Prathibhani *et al*., 2021). Moreover, there is no published work on the morphology of *Cinnamomum* bark powder, although it is also important in identifying adulterations.

A few molecular-based analyses conducted over the last two decades are also available (P D Abeysinghe *et al*., 2014; Pushpa Damayanthi Abeysinghe *et al*., 2009; Swetha *et al*., 2014). For example, Abeysinghe and colleagues used Random Amplified Polymorphic DNA (RAPD) and Sequence Related Amplified Polymorphic markers (SRAP) for the identification of wild relatives (P D Abeysinghe *et al*., 2014). Another study by the same scientists suggested that the chloroplast regions of the *trnL* intron, the *trn*T-*trn*L, *trn*L-*trn*F, and *trn*H-*psb*A intergenic spacers, and the internal transcribed spacer (ITS) of ribosomal DNA could not differentiate the wild relatives of *Cinnamomum* at the intra species level (Pushpa Damayanthi Abeysinghe *et al*., 2009). However, these studies consisted of only a few species, namely, *C. dubium*, *C. capparu-coronde*, *C. rivulorum*, *C. citriodorum*, and *C. litseifolium.* A recent study utilized universal chloroplast barcoding regions; *rbcl, matK,* and *trnH-Psb1* concluding that the nucleotide diversity in the considered regions could not discriminate wild *Cinnamomum* species in Sri Lanka (Chandrasekara *et al.*, 2021). The polymorphic Inter Simple Sequence Repeats (ISSR) regions could differentiate the closely related species, *C. verum - Sri Gamunu* and *Sri Wijaya,* and the individuals of an open-pollinated progeny. As such, it would be important to look at the possibility of using ISSR for genetic diversity analysis of *Cinnamomum* species.

Therefore, the current study included an ISSR analysis and the bark powder morphology of all *Cinnamomum* species found in Sri Lanka. Further, floral morphology, including the Scanning Electron Microscopy of pollen

grains, was assessed to identify cultivated and several other wild *Cinnamomum* species.

MATERIALS AND METHODS

Research location and genetic materials

The samples were collected, as previously described by Chandrasekara *et al.*, (2021). Except for *C. ovalifolium,* the other samples were collected from the same agro-ecological zone, along with sample voucher numbers and GPS coordinates (Table 1).

DNA extraction

Total genomic DNA from leaf samples was extracted using the SDS method with minor modifications (Pathirana *et al*., 2018). Leaf samples were ground using liquid nitrogen to a fine powder, and 200 mg of each was added to 750 µL of lysis buffer and vortexed for 1 min. *C. sinharajaense*, *C. dubium*, and *C. citriodorum* samples were incubated at 65 ^{o}C overnight, and the others were incubated for around one hour while inverting the tube every 10 minutes. After centrifuging the samples for 10 minutes at 13,000 rpm, the supernatant was added to 5M ½ volume of NaCl and centrifuged for 10 minutes at 13,000 rpm. Then, 500 µL of ice-cold isopropanol was added to the supernatant and left for 5 min at room temperature, then centrifuged for 10 min at 13,000 rpm. After washing with 500 µL of 70% ethanol, the DNA pellet was air-dried and re-suspended in 30 µL of nuclease-free water. DNA was confirmed by spotting genomic DNA on a 0.8% agarose gel and visualizing it under UV light. The quality and quantity of DNA samples were assessed with agarose gel electrophoresis and NanoDrop spectrophotometer (Nano2000, Thermo Scientific, Wilmington, Delaware, USA).

Table 1. *Cinnamomum* species used in the study and their sampling locations

Species	Passport number	Location	GPS coordinates
Cinnamomum capparu-coronde (C1)	DE.C1	MRS, *Dalpitiya*	7°08'04.6"N 80°35'06.4"E
Cinnamomum citriodorum (C2)	MA.C2	NCRTC, *Matara*	5°58'54.64"N 80°33'42.8"E
Cinnamomum dubium (C3)	DE.C3	MRS, *Dalpitiya*	7°08'00.2"N 80°35'08.2"E
Cinnamomum litseifolium (C4)	DE.C4	MRS, *Dalpitiya*	7°07'57.0"N 80°35'16.4"E
Cinnamomum ovalifolium (C5)	HP.C5	HPNP, *N'eliya*	6°48'12.6"N 80°48'11.2"E
Cinnamomum rivulorum (C6)	MA.C6	NCRTC, *Matara*	5°58'54.64"N 80°33'42.8"E
Cinnamomum sinharajaense (C7)	MA.C7	NCRTC, *Matara*	5°58'54.64"N 80°33'42.8"E
Cinnamomum verum (*Sri Gamunu*) (C10)	MA.C10	NCRTC, *Matara*	5°58'54.64"N 80°33'42.8"E

Agro-ecological zones are expressed as previously described by Panabokke (1975)

Table 2: UBC ISSR Primers and their melting temperatures

Primers	Sequence 5' to 3'	Melting Temperature (Tm) ^{o}C
UBC808	AGA GAG AGA GAG AGA GC	48.8
UBC834	AGA GAG AGA GAG AGA GYT	49.2
UBC835	AGA GAG AGA GAG AGA GYC	49.5
UBC840	GAG AGA GAG AGA GAG AYT	48.5
UBC841	GAG AGA GAG AGA GAG AYC	48.3
UBC842	GAG AGA GAG AGA GAG AYG TCC C	47
UBC864	ATG ATG ATG ATG ATG ATG	43
UBC888	CAC CAC ACA CAC ACA CA	52.4

PCR amplification

Polymerase chain reaction (PCR) amplifications were performed in a 25 µL reaction volume containing 2.0 µL of 30 ng of template DNA, 0.4 µL of 200 mM dNTPs, 5µL of 5X Green go Taq buffer, 1.5 µL of 25 mM $MgC1_2$, 0.1 µL of Go Taq Flexi DNA polymerase (Promega, Cat No: M8295), 1 µL of 10 µM UBC ISSR primer, 2 µL of PVP (10%), 3 µL of 10 mM Spermidine, and 10 µL of sterile distilled water using a Veriti Thermal Cycler (Applied Biosystems, USA). The PCR amplification was performed under the following cycle profile: 5 min at 94 ^{0}C, followed by 1 minute at 94 ^{0}C, 30 s annealing temperature of each primer, 1-minute extension at 72 ^{0}C for 30 cycles, and 5 minutes at 72 ^{0}C for the final extension. The annealing temperature was adjusted according to the primer's melting temperature (Tm) (Table 2) used in the reaction.

Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) was performed for an effective base pair separation for the identified polymorphic primers. Eight percent (8%) Acrylamide gel was prepared to separate 60-400 bp range. Polyacrylamide gels were prepared with 3.9 ml of 30% Acrylamide, 3 ml of 5X TBE, 105 µl of 10% Ammonium Persulphate (APS) solutions, 7.9 ml of sterile water, and 5.25 µl of TEMED (N,N,N',N' – tetramethylenediamine). The gels were visualized under a UV gel documentation system (Chemi Doc TM XRS+ Molecular Imager, Bio-Rad Laboratories Inc., Hercules, California, USA) and photographed.

Statistical analysis

Well-separated and reproducible bands were scored as discrete variables using '1' and '0' to indicate the presence and absence of bands, respectively. Those binary data were used for inter-population and group analysis using the software POPGENE32 (Francis and Yang, 2000, cited in Attanayake *et al.*, 2017). The number of total bands (TB), number of polymorphic bands (PB), and percentage of polymorphic bands (PPB) were calculated for each primer. Marker performance was assessed from polymorphic information content (PIC) was calculated as:

PIC = 2*f*(1-f) (f=number of polymorphic bands)

The binary data for eight samples was converted into a distance matrix by the Dendro UPGMA (Garcia-Vallve and Puigbo, 2009) program with 100 bootstraps. Phylogenetic analyses were done with the PHYLIP-3.695 phylogeny inference package (Felsenstein, 2002). The consensus unweighted pair group method with arithmetic averages (UPGMA) deprogram was generated, viewed, annotated, and printed using FigTree (Rambaut, 2009).

Powder Microscopy

All the collected samples were shade dried, coarsely powdered with the help of a grinder, and sieved through sixty mesh sieves, which is similar to commercial scale production (International Basic Safety Standards (BSS)-60, American Society for Testing and Materials (ASTM) – 60, ISO -250-micron aperture size), to size separate and used for microscopy studies. Moreover, 8 mesh sieves (International Basic Safety Standards (BSS)-08, American Society for Testing and Materials (ASTM) – 10, ISO -2057 micron aperture size) was also used to compare the fibers of *C. verum* powder, The powdered samples were soaked in 70% commercial Clorox detergent for 20 minutes. The samples were then washed with distilled water. Slides were prepared by staining the soaked spice powders with safranin and observed under a microscope (Olympus LED Trinocular microscope) and the images were captured.

Flower morphology screening

The collected flowers were morphologically evaluated using six floral morphological characters, based on the identification of Ravindran *et al.,* (2004). Table 3 represents the four quantitative and two qualitative characters that were considered. All the measurements were collected as described in the cinnamon descriptor TURIS (TURIS, 2013).

Table 3: Qualitative and quantitative floral characteristics of Cinnamomum

No	Traits	Description
01	Flower colour (PC)	1- Greenish-white, 2- Yellowish-green, 3- Yellowish-white, 4- White
02	Petal pubescence (PP)	Distribution: 1- Dense, 2- Intermediate, 3- Sparse; Color of pubescence: 1- Silvery, 2- White, 3- Yellowish
03	MNFP	Maximum number of flowers per panicle, Maximum fully open flowers available in panicle from 9 am to 12 pm
04	MLP	The maximum length of available panicle; from top most flower to attachment in the branch (mm)
05	APdL	Average pedicel length; Average of 5 flowers from the beginning of pedicel to flower attachment
06	APeL	Average petals length; an average of ten petals from the tip of the petals to the attachment

Scanning Electron Microscopy

The fresh anthers were separated from the flower and fixed in FAA solution, 5% formaldehyde (v/v), 5% (v/v) acetic acid, and 45% (v/v) ethanol for long-term preservation. The preserved fresh pollen was washed twice in distilled water for 15 minutes. Then the pollen was dehydrated in 100% Ethanol using an ethanol series followed by transferring to 100% Hexamethyldisilazane (HMDS) through a graded series and soaked for 20 minutes at each stage. The fully dried pollen was mounted onto the sample stub using carbon tapes and gold sputter-coated for 15 seconds. The coated samples were examined by Hitachi SU6600 Analytical Variable Pressure FE-SEM (Scanning Electron Microscope) at the Sri Lanka Institute of Nanotechnology (SLINTEC), Homagama, Sri Lanka.

RESULTS & DISCUSSION

Species delimitation in complex groups and those with varying degrees of morphological overlap is a tedious and difficult task. Under the said circumstances, it is recommended that different and combined approaches, such as morphological, molecular, and cytological methods, be used to determine species boundaries.

All the species included in the study are endemic to Sri Lanka. However, only *C. verum* has been cultivated and used widely in the country (Senaratne *et al*., 2020). One study has focused on using molecular methods along with morphometric methods to identify and find phylogenetic affinities in *Cinnamomum* in Taiwan for taxonomical and conservation purposes, respectively (Ho *et al*., 2011). Only a few reported attempts have been made to assess the diversity of Sri Lankan *Cinnamomum* using nuclear markers. (P D Abeysinghe *et al*., 2014) used RAPDs and SRAP markers to assess the inter-species and intra-species variation. A recent study showed that the nucleotide diversity in the universal barcoding regions could not resolve the phylogeny of Sri Lankan *Cinnamomum* wild species (Chandrasekara *et al.*, 2021).

Identification of polymorphic markers

This is the first report on using ISSR markers in surveying genetic variation and determining genetic relationships in the genus *Cinnamomum*. Eight *Cinnamomum* species including *C. verum* were studied using ISSR markers, which were selected based on their relative technical simplicity, robustness, and a higher level of polymorphism detected in *Cinnamomum* (Liyanage, *et al*., 2020). The ISSR regions, UBC 808, 834, 835, 840, 841, 842, 864, and 888 from the University of British Columbia (UBC) were used for the analysis (table 4).

Resulted polyacrylamide PAGE images of the study are shown in Figure 1. The highest polymorphic percentage was shown by the UBC primer 888 followed by UBC 840 (88.9%), UBC 834 (85.7%) and UBC 842 (73.9%) (Table 5). UBC 808 and UBC 864 did not show polymorphism among wild species. For the eight individuals included in the analysis *of C. verum* and seven wild species, four primers produced a total of 73 amplified bands, of which 61 (83.5%) were

polymorphic and 12 (16.5%) were monomorphic. The ISSR analysis of ten individuals from the same pollination event of cultivated *Cinnamomum* showed 80.53% polymorphism when the same primers were used (Liyanage *et al*., 2020). Generally, the performance of ISSR markers and their informativeness are assessed with using the PIC value. The average PIC value of 0.384, observed in this study, is closer to 0.5, which is the maximum value of PIC for any dominant marker (Nagy *et al*., 2012). The high PIC values obtained for ISSRs reflect the efficiency of the marker to simultaneously analyze a large number of bands rather than the level of polymorphism detected (Powell *et al*., 1996). Accordingly, UBC 888, 834, and 840 were considered suitable regions for analysis of species-level variation in *Cinnamomum.*

Table 4: Polymorphism of selected ISSR markers

Wild relatives	Polymorphism/Monomorphism							
	808	**834**	**835**	**840**	**841**	**842**	**864**	**888**
C. capparu-coronde	0	0	0	1	0	1	0	1
C. citriodorum	0	0	0	1	0	1	0	1
C. dubium	0	0	1	1	0	1	0	1
C. litseifolium	0	0	1	0	0	1	0	1
C. ovalifolium	0	0	1	1	0	1	0	0
C. rivulorum	0	1	0	0	0	1	0	1
C. sinharajaense	0	1	0	1	0	1	0	0
C. verum	1	1	1	1	1	1	1	1

Note: Polymorphic primers "1", monomorphic primers "0"

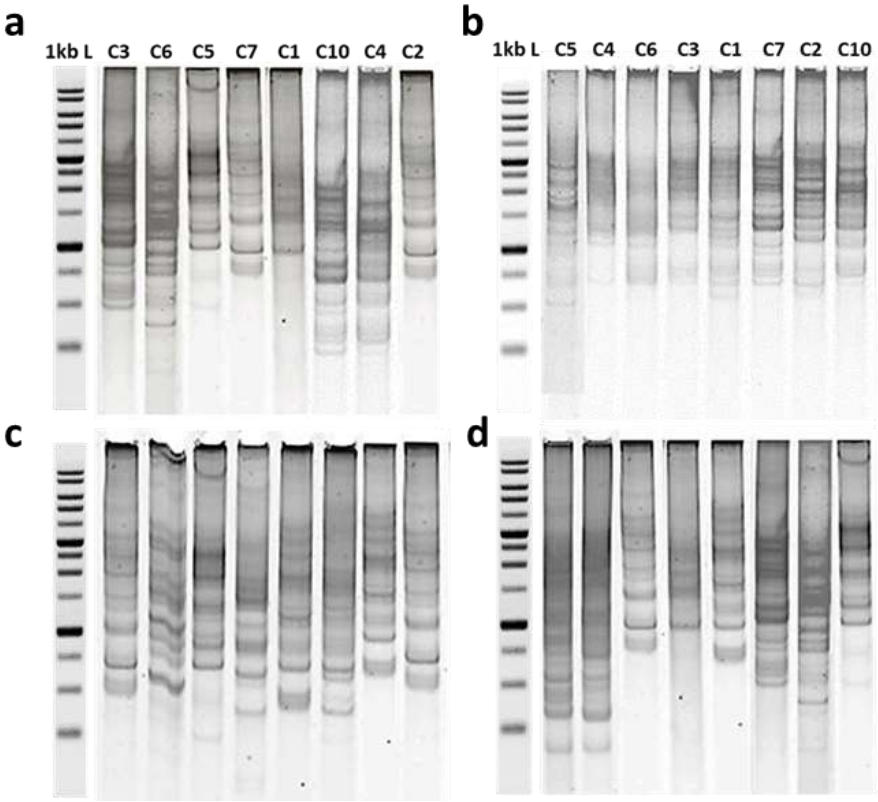

Figure 1: PAGE plates of wild and cultivated species of genus *Cinnamomum.* (a) 888, (b) 842, (c) 834, (d) 840. Note: L= 1 kb ladder, C1=*C. capparu-coronde*, C2=*C. citriodorum*, C3= *C. dubium* C4=*C. litseifolium*, C5=*C. ovalifolium*, C6= *C. rivulorum*, C7= *C. sinharajaense,* C10=*C. verum*

Table 5: ISSR polymorphism of the genus Cinnamomum.

Primer code	Number of loci			*ppb* (%)	*PIC*	*H*
	Monomorphic	Polymorphic	Total			
UBC 834	2	12	14	85.7	0.468	
UBC 840	2	16	18	88.9	0.500	
UBC 842	6	14	20	73.9	0.193	
UBC 888	2	19	21	90.5	0.375	
Population-level	12	61	73	83.6	0.384	0.3128

Note: *ppb*=percentage of polymorphic bands, *H*= Nei's genetic diversity

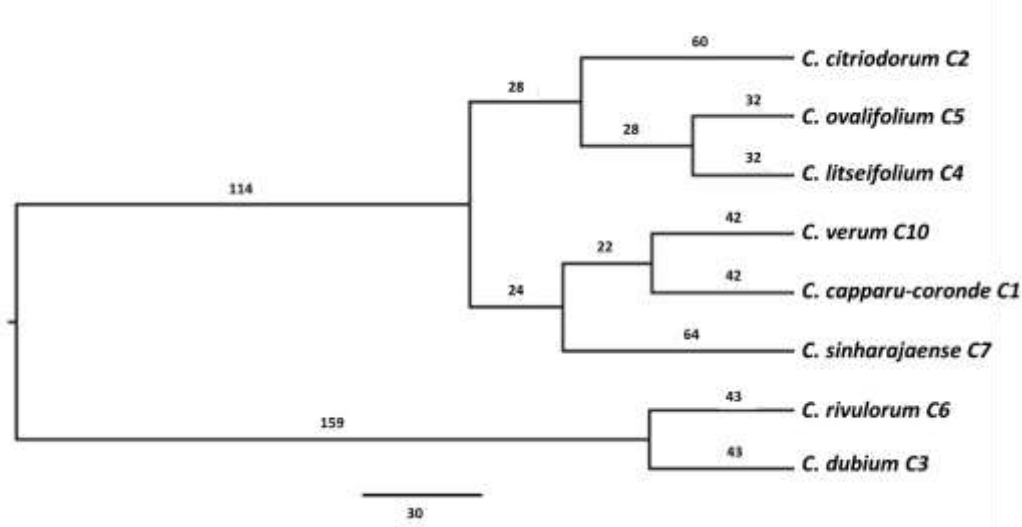

Figure 2: Genetic relationship of the genus *Cinnamomum* in Sri Lanka based on ISSR markers.

The UPGMA tree depicted through ISSR markers grouped *Cinnamomum* into two main clusters, similar to the leaf morphology analysis (Bandusekara *et al*., 2020). As a result*, C. dubium* and *C. rivulorum* clustered separately, while the other cinnamon species clustered together, except for *C. capparu-coronde, C. verum*, and *C. sinharajaense,* which clustered closely (Figure 2). However, the genetic diversity analysis based on *psbA-trnH* and matK barcoding regions resulted in a different relationship among Sri Lankan *Cinnamomum* species (Chandrasekara *et al*., 2021).

Cluster analysis was performed by using four polymorphic UBC ISSR primers: 834, 842, 840, and 888. Scored bands were tested in the consensus unweighted pair group method with arithmetic averages (UPGMA), the dendrogram was generated and printed using the software "FigTree".

Biparentally inherited ISSRs are amplified from both the nuclear and organellar regions, whereas maternally inherited barcoding regions such as *rbcL*, *matK*, and *psbA-trnH* are amplified from the cpDNA. The nucleosome is large in comparison to the plastome and the chondriome. As a result, the origin of ISSR polymorphism is most likely nuclear, and it can serve as a control for the groups found in the plastid region. Due to haploid and uniparental inheritance, cpDNA only reveals half the parentage of plants with hybridization or polyploidy, and thus plants can be incorrectly classified into a clade of one of two parents (Ma *et al.,* 1999). As a result, phylogenetic trees based on cpDNA may be contradictory (Treutlein *et al*., 2003). However, the current ISSR analysis could not differentiate closely related species with sufficient statistical confidence (Figure 02). Nei's genetic diversity classification of H=0.3128 between the cultivated species *C. verum*, and the wild species (as a group), is not high suggesting a low level of diversity between cultivated and wild species, and moreover it is evident from the phylogenetic analysis as well.

Microscopic analysis of powder

The fiber and scleroid cell quality were assessed using light microscopic images (Figure 03). The average lengths of fibers from different samples were recorded as; 1129±266.2 µm in *C. verum*, 970±234.4 µm in *C. ovalifolium*; 219.4±97.2 µm in *C. dubium,* 439.4±102.3 µm in *C. citriodorum,* 522.3±73.4 µm in *C. rivulorum,* 345.2±45.5 µm in *C. capparu-coronde* and 383.8±53.2 µm in *C. litseifolium*. The fibers were observed to be mostly narrow and elongated cells with tapering ends, which could be easily identified from tracheid cells. The secondary thickening of the fibers was high in *C. verum, C. ovalifolium*, as well as in *C. capparu-coronde.* Accordingly, *C. verum* and *C. ovalifolium* have longer fibers, while *C. dubium and C. rivulorum* have shorter fibers (Figure 3). The data suggests that the cinnamon powder quality could be used in species identification to a certain extent. Such a simple analysis can be extended to identifying the adulterants of exporting materials which is considered a serious issue with *C. verum* in the industry (Jeremić *et al*., 2019).

Generally, we use the bark of the Cinnamon tree (Quillings) to process Cinnamon powder. According to the Spice Capacity Chart, three types of ground cinnamon are manufactured: Superfine Cinnamon Powder (60 mesh), Normal Cinnamon Powder (40 mesh), and Cinnamon Powder no. 1 (30 mesh). If a higher mesh size (30 mesh or above) is used, it will improve the characteristics of processed cinnamon.

In order to obtain the finest fiber for our examination, we utilized a sieve with a mesh size of 60, ut and we also used an 8-mesh sieve to demonstrate that the mesh size had no bearing on the quality of fiber. In commercial scale cinnamon processing, the grinding is done at high speed using a grinder or in a mill, while the same strategy has been used in the current study. The finest powder was subjected to microscopy analysis. Both grades of powdered cinnamon had cinnamon fibers that were primarily narrow and elongated cells with tapering ends. As a result, both commercially and domestically, adulteration from any other low-grade cinnamon species or other plant species might be easily detected.

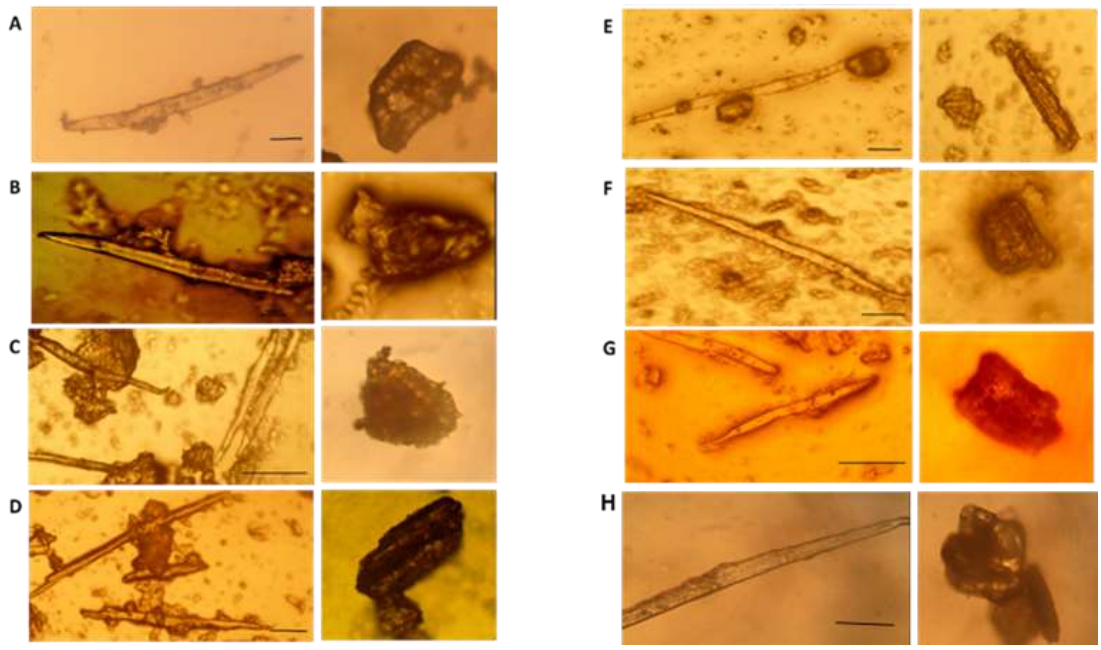

Figure 3: Powder Microscopy of *Cinnamomum* species for xylem fiber and stone cell. (A-G) powder obtained from 60 sieve mesh (A)- *C. verum* (B)- *C. ovalifolium* . (C)- *C. dubium*, (D)- *C. citrodorum*, (E)- *C. rivulorum*, (F)- *C. capparu-coronde* (G)- *C. litseifolium* (1 bar= 100 µm) (H) *C. verum* (8 sieve mesh)

Floral Morphometric analysis

The cinnamon flowers are small, cream to white, with nine functional and three non-functional stamens. Functional stamens are arranged as three inner whorls and six outer whorls (Figure 4). The petal color of the flower (PC) varies from white to greenish and yellowish, while the petal pubescence (PP) color varies from white to silver grey. The maximum length of the panicle (MLP), average panicle length (APdL), and average petal length (APeL) vary among species from 12 mm to 286 mm, 2.85 mm to 5.45 mm, and 1.21 mm to 3.66 mm, respectively. The maximum number of flowers per panicle among the observed samples varies from 2 to 14. Both flowers of *C. ovalifolium* and *C. litseifolium* show similarities in PC, MNFP, APdL, and PP; with *C. dubium* and *C rivulorum* showing the same. *C. sinharajaense* was the largest flower with 3.48 ± 0.28 in APL with unique silvery grey color PP. Further, both *C. capparu-coronde* and *C. sinharajaense* show less PP than the other six species. *C. verum* was found to be the largest panicle (Table 6).

Table 6: Flower characteristics of genus *Cinnamomum* in Sri Lanka.

Species	PC	MNFP	MLP (mm)	APdL (mm)	APeL (mm)	PP
C. capparu-coronde	White	3-5	128.6	5.45±0.75	1.83±0.23	Intermediate white
C. dubium	Greenish white	6-8	64.5	5.52±0.94	2.65±0.31	Densely white
C. litseifolium	Yellowish white	2-3	62.6	2.45	1.58±0.15	Densely silvery
C. ovalifolium	Yellowish white	2-3	38.6	2.85	1.42±0.21	Densely silvery
C. rivulorum	Greenish white	8-10	66.8	3.94±0.85	2.43±0.15	Densely white
C. sinharajaense	White	3-5	110.8	5.51±0.31	3.48±0.28	Intermediate silvery gray
C. verum	White	12-14	286.8	4.56±0.89	2.83±0.83	Densely white

Notes: (PC) Flower color; greenish-white, yellowish-green, yellowish-white, white, **(PP)** petal pubescence; dense, intermediate, sparse, color of pubescence; silvery, white, yellowish, **(MNFP)** Maximum number of flowers per panicle; Maximum fully open flowers available in panicle, **(MLP)** Maximum length of available panicle; from top most flower to attachment in the branch, **(APdL)** Average pedicel length; from the beginning of pedicel to flower attachment, **(APeL)** Average petals length; average of ten petals from the tip of the petal to attachment.

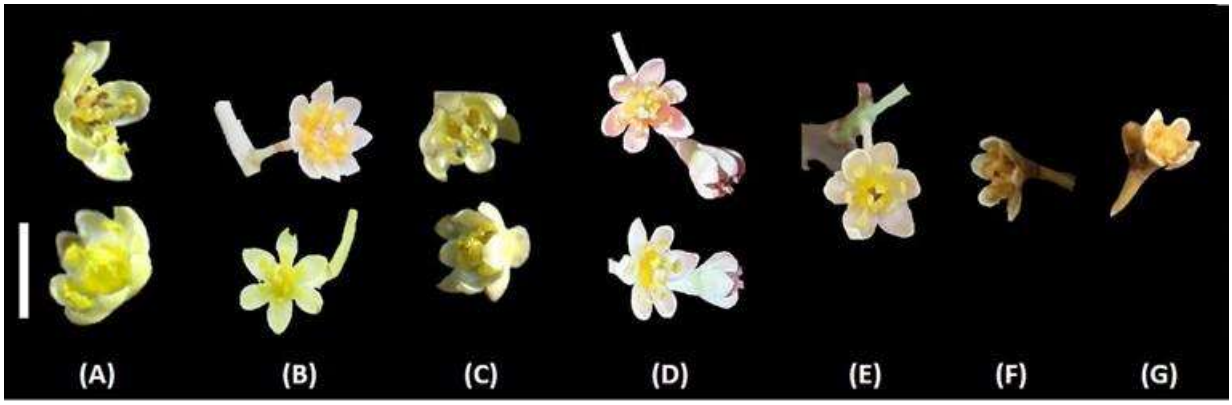

Figure 4. Representative flower samples of cinnamon wild and cultivated species.(A)- *C. sinharajaense* (B)- *C. verum*. (C)- *C. dubium*, (D)- *C. capparu-coronde*, (E)- *C. rivulorum*, (F)- *C. litseifolium*, (G)- *C. ovalifolium*, Bar = 1 cm. All flowers were at their fully open stage.

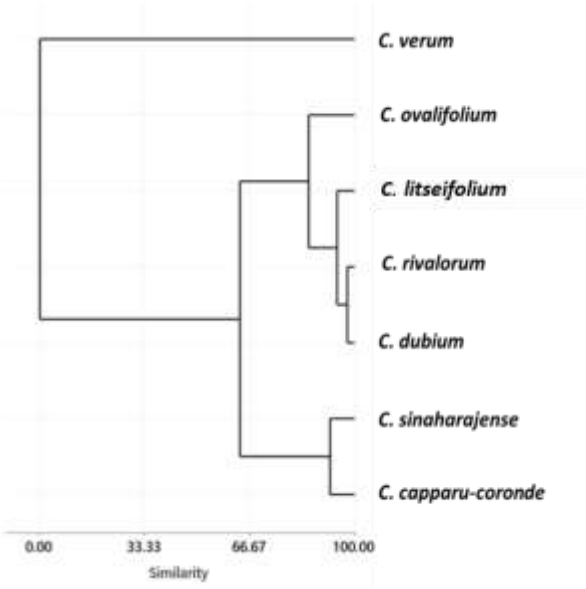

Figure 5: Phylogenetic tree of seven cinnamon species of genus *Cinnamomum* in Sri Lanka
Dendrogram was drawn using qualitative and quantitative floral characteristics. Complete linkage method and Manhattan distance matrix generated using Minitab v 19.2020.1
[Qualitative data scale: PC- white (0), Yellowish white (1), Greenish white (2). PP: Densely white (0), Intermediate white (1), Densely silver (2), Intermediate silvery gray (3)]

According to the UPGMA cluster analysis (Figure 5) of floral morphology, wild *Cinnamomum* species were divided into two major groups. *C. capparu-coronde and C. sinharajaense* were clustered together at a similarity level of 92.20, while *C. verum* was out clustered since the size of the flower is larger than others. The other four species clustered in another group at a similarity level of 85.45, while *C. dubium* and *C. rivulorum* clustered at a similarity level of 97.71. To some extent, the floral morphological dendrogram was supportive of the molecular cluster analysis. For example, *C. rivulorum and C. dubium* clustered together in the molecular and floral morphology analyses. Although *C. verum* clustered with *C. capparu-coronde* and *C. sinharajaense* in molecular analysis, *C. verum* clustered separately in morphology analysis.

Pollen morphology

A description of the pollen grain morphology of five *Cinnamomum* varieties under analysis is illustrated in the SEM photographs (Figure 6). The morphological observations for the quantitative and qualitative features are summarized in Table 7. The pollen grains of the investigated *Cinnamomum* varieties are all spheroidal in shape, apolar, and inaperturate. The systematic value distinguishes pollen morphology, which varies within species, and is thus useful in identifying *Cinnamomum* at the species level. According to the PCA done at a preliminary level, the pollen size, length of spines, inter-spinular distance, and spine-ornamentation significantly contributed to the species variation (Figure 7). Therefore, these characteristics need to be studied comprehensively, including all the species and more samples from each species.

Nevertheless, floral morphology data support the genetic relationships suggested by the ISSR analysis markers. For example, *C. dubium* and *C. rivulorum* clustered together in both analyses.

As such, the combination of morphological, molecular, and cytological methods could clearly differentiate some species, such as *C. verum, C, ovalifolium* and *C. litseifolium*, while others such as *C. sinharajaense* and *C. capparu-coronde* could not. Most of the

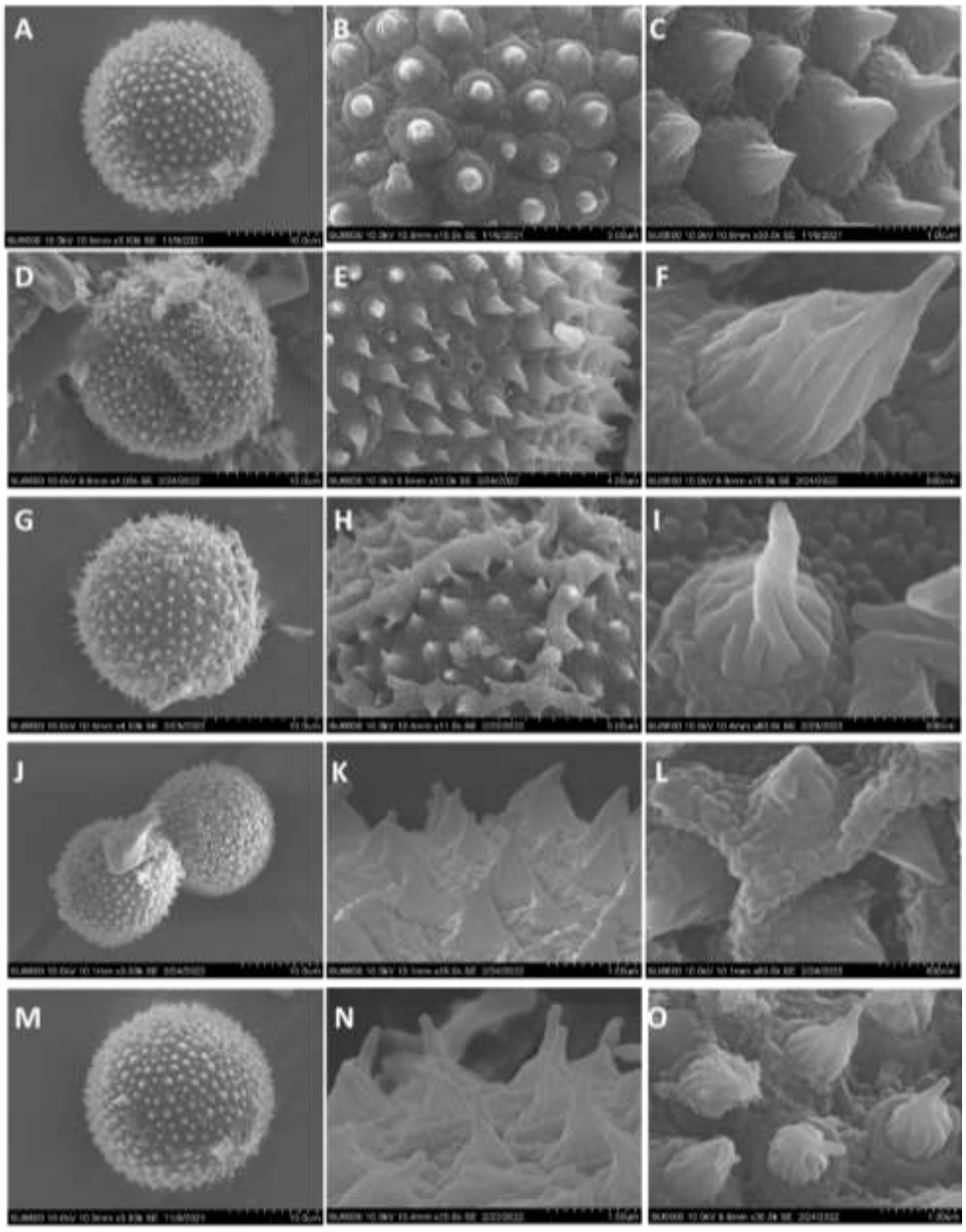

Figure 6: Scanning electron microscopy (SEM) images of pollen grains of *Cinnamomum*
(A,B,C) Cinnamomum verum (A) Polar view of pollen grains (B) detail of spines and exine structure /Exine ornamentation (C) Dorsa-Ventral view of the enlarged spine, striated conical shaped spine on a cushion base. (D,E,F) *Cinnamomum capparu-coronde* (D) Polar view of pollen grains (E) detail of spines and exinestructure /Exine ornamentation, tectum is less perforated (F) Ventral view of an Straited Conical linear spine on a cushion base. (G.H.I) *Cinnamomum dubium* (G) Polar view of pollen grains (H) detail of spines and exine structure /Exine ornamentation, tectum is highly perforated (I) Ventral view of an Straited spine on a cushion base. (J,K,L) *Cinnamomum rivulorum* (J) Polar view of pollen grains (K) detail of spines and exine structure /Exine ornamentation, tectum is perforated (L) Ventral view of an Straited Conical linear spine on a cushion base. (M,N,O) *Cinnamomum ovalifolium* (M) Polar view of pollen grains (N) detail of spines and exine structure /Exine ornamentation,(O) Ventral view of an Straited spine on a cushion base

Table 7: Quantitative and qualitative pollen morphological characters of *Cinnamomum*

	PL ± SD (P) µm	**LS±** SD **µm**	**ID±** SD **µm**	**Tip of spines**	**Spine ornamentation**	**Granule Type**	**Nature of granules**	**Nature of cushion base**
Cinnamomum verum	24.36±0.123[a]	1.11±0.113	0.48±0.103	Acute-acuminate	Striated	Monomorphic	Prominent	Prominent
Cinnamomum capparu-cornode	21.54±0.598 [a]	0.88±0.011	1.01±0.073	Acuminate-acute	Striated	Monomorphic	Prominent	Prominent
Cinnamomum dubium	18.06±0.22 [b]	1.05±0.064	0.66±0.090	Acuminate-acute	Striated	Monomorphic	Prominent	Prominent
Cinnamomum rivulorum	18.91±0.23 [b]	0.87±0.01	1.56±0.01	Acuminate-acute	Striated	Heteromorphic	Prominent	Compactly arranged
Cinnamomum ovalifolium	22.04±0.42 [a]	0.92±0.15	1.12±0.23	Acuminate-acute	Striated	Heteromorphic	Sparsely distributed	Spatially arranged

PL- Polar length, LS- length of the spinule, ID- Interspinular distance
*Significant at α=0.05

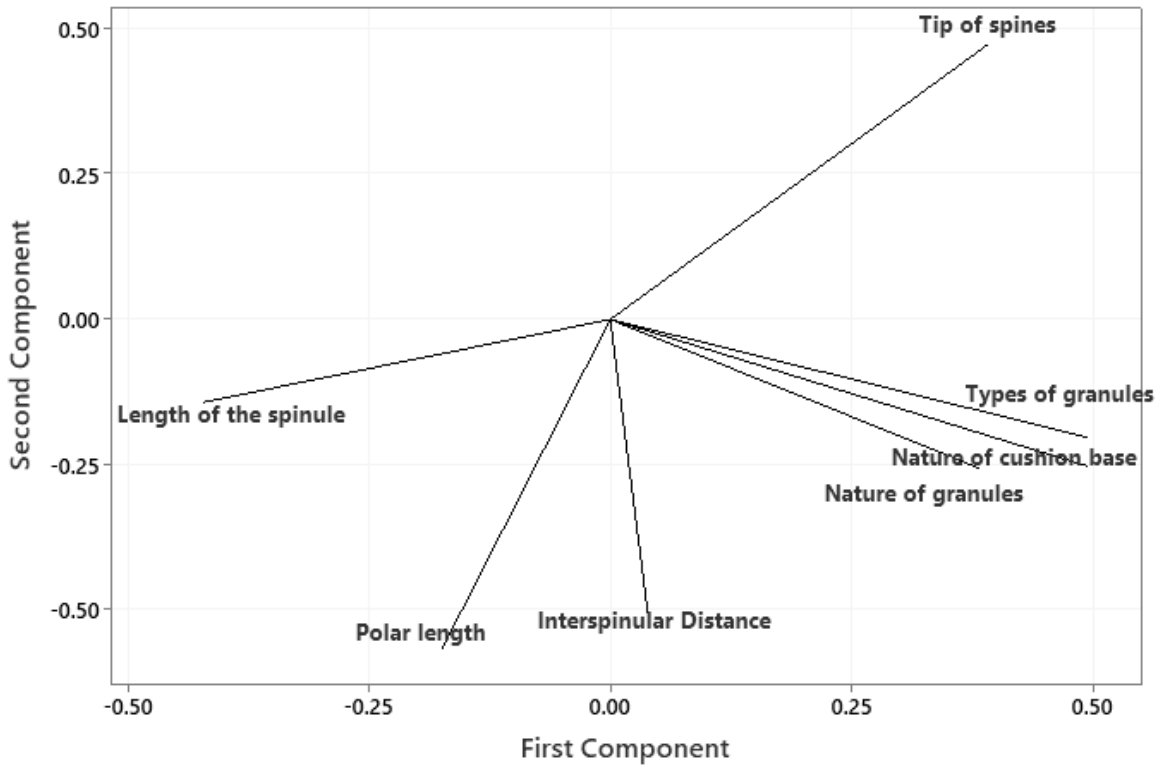

Figure 7: Principal component Analysis (PCA) of qualitative and quantitative characters of the pollen morphology

possible morphological and biochemical features of *Cinnamomum* species in Sri Lanka have already been captured in previous studies (Bandusekara *et al*., 2020; T. Liyanage *et al*., 2017; Prathibhani *et al*., 2021) and the current study. Therefore, further studies based on comprehensive genome analysis would be needed to resolve the phylogeny of these closely related species.

CONCLUSIONS

Though several polymorphic ISSR regions were identified, ISSR marker analysis could not reliably distinguish between closely related species with sufficient statistical confidence. The size and shape of the bark fiber differ between the wild and cultivated species and could be used as a key to identify adulterants during export. The flowers of the domesticated cinnamon species, *C. verum,* and the two wild species; *C. sinharajaense,* and *C. capparu-coronde*, are significantly larger than those of other species. Flowers may distinguish between species *C. sinharajaense* and *C. capparu-coronde* based on their colour and shape. According to the SCM analysis, size of pollen, length of spines, distance between spines, and the ornamentation on spines were all major contributors to species-level diversity. Such variations require in-depth examination, including all the species. Nevertheless, the ISSR regions, bark powder morphology, and floral morphological features assessed could not resolve the phylogeny of *Cinnamomum* species found in Sri Lanka, and further studies are suggested.

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