

Development of Species-Specific ISSR-Derived SCAR Marker for Early Discrimination Between *Cinnamomum verum* and *Cinnamomum cassia*

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Abstract

Background *Cinnamomum verum* (true cinnamon) and *Cinnamomum cassia* (cassia cinnamon) are two important species belonging to family Lauraceae. These species are recognized by morphological, chemical composition and essential oil contents. The appropriate identification of species would be considerably improved by a genetic method. The main objective of the present study was to develop molecular markers distinguishing between *C. verum* and *C. cassia*.

Methods and results A total 71 ISSR (Inter simple sequence repeat) and four universal barcoding (*ITS*, *rbcL*, *matK*, and *psbA-trnH*) genes were used to distinguish both the species. No sequence variation was observed between the two species for any DNA barcode gene. However, one ISSR i.e. ISSR-37 showed a clear distinction between the species and produced 570 bp and 746 bp amplicons in *C. verum* and *C. cassia*, respectively. The polymorphic bands were converted into species specific SCAR markers. The SCAR-CV was specific to *C. verum* and amplified 190 bp band, however there was no amplification seen in the *C. cassia* samples.

Conclusion The SCAR primers generated in this study can be employed as efficient, economical, and reliable molecular markers for the identification of *C. verum*.

Introduction

Cinnamon (*Cinnamomum* spp.), belongs to the family Lauraceae, is one of the earliest recognised spices growing in Africa, Southeast Asia, China, and Australia. *Cinnamomum* has long been valued for both its flavour and therapeutic benefits. *Cinnamomum* is a genus of tiny, 10–15 m tall, evergreen trees and shrubs. The leaves are pinnately in morphology and vary in length from 7 to 18 cm. The bark is frequently used as a spice (Cardoso-Ugarte et al. 2016). The genus *Cinnamomum* includes four important economically valuable species of cinnamon including- *C. verum*, *C. cassia*, *C. loureiroi* and *C. burmannii*. Among these, *C. verum* is known as real cinnamon, Ceylon or Sri Lankan cinnamon. The other three spp of *Cinnamomum* are commonly known as cassia Cinnamon (Killday et al. 2011) due to their similarity in appearance, texture, and coumarin quantity. Cinnamon contains metabolite coumarin which is harmful to human health. A high coumarin level increases the risk of liver damage or failure (Lungarini et al. 2008). True cinnamon only has a trace amount of coumarin about 0.004% in contrast to the up to 1% found in cassia cinnamon. The morphologically similar cassia cinnamon, which has a rougher, thicker, less expensive, and less scented bark, is frequently used to adulterate true cinnamon (*C. verum*) barks. Consequently, it is essential to distinguish between these two main categories and recognize food adulteration (Abeyasinghe et al. 2009).

Earlier, plant identification relied on morphological, microscopic and biochemical investigation, which was frequently insufficient to accurately identify a plant species due to high influence of environmental factors. Recent advances in molecular identification methods using DNA markers which are distinctive, reliable, and pervasive to plants have been made to address these issues. Using DNA based molecular markers to assess genetic variation in plants has demonstrated advantages; they are unique, neutral, feasible, less expensive, unaffected by physiological condition, unrelated to age and tissue type and even more informative than morphological markers. Therefore, DNA fingerprinting is being deployed for plant varietal identification (Kumar et al. 2014). Among DNA markers, DNA barcoding is considered as the most effective approach for species classification and identification. DNA barcoding is a well-known method for identifying species by employing

short, regular DNA sequences as markers. DNA can be taken out of mitochondria, chloroplasts, or nuclei (Hebert et al. 2003). DNA barcoding genes are commonly drawn from six major candidate regions including internal transcribed spacer (*ITS* and *ITS2*), ribulose-1,5-bisphosphate carboxylase (*rbcL*), maturase K (*matK*), photosystem II protein D1-tRNA-His (*trnH*) and cytochrome c oxidase I (*COI*) (Tehen et al. 2014). This method is extensively used for testing, validation and identification of medicinal plants.

Several molecular markers, including RAPD (Random amplified polymorphic DNA), AFLP (Amplified fragment length polymorphism), SSRs (simple-sequence repeats), and ISSR (inter simple sequence repeats) can also be used to identify different species (Bi et al. 2021, Hadipour et al. 2020, Yang et al. 2022). Inter simple sequence repeat (ISSR), one of the molecular marker which is nowadays mostly used. ISSR marker provides rapid, accurate and highly informative approach. Mendelian inheritance is used for ISSR markers, which segregate as dominant markers. There is no prior sequence knowledge is required for ISSR fingerprinting (Nybom et al. 2014). This technique is effective because it has been used in numerous studies to characterize genetic stability assessment (Tikendra et al. 2021), cultivars identification (Nagaranju et. 2002), genome mapping (Gupta et al. 2012), genetic diversity (Hadipour et al. 2020) and phylogenetic relationship within species (Rameshkumar et al. 2019). The ISSR marker is quite confined because of its poor reproducibility and sensitivity towards different experimental conditions. In an effort to improve its reproducibility, the SCAR (sequence characterized amplified regions) marker was developed. SCAR markers are PCR-based codominant marker, more effective, locus specific and more reproducible commonly produced from RAPD and ISSR fragments (Kiran et al. 2010, Cheng et al. 2016). SCAR markers have become useful tools for differentiating across species that share similar morphology and phenotype in various plant species including *Chrysanthemum morifolium* (Cai et al. 2022), *Moringa oleifera* (Ravi et al. 2021), *Camellia sinensis* (Xu et al. 2019), *Lycium chinense* (Liu et al. 2020) and *Acer rubrum* (Boyd et al. 2019). Additionally, SCAR markers have reported their role in sex identification of early stage plant (Korekar et al. 2012), authentication of various medicinal herbs (Kiran et al. 2010), selection of hybrid strain (Wang et al. 2011) and identification of genes that resist powdery mildew (Srivastava et al. 2012).

In view of these, the present study focused to develop a faster approach for discrimination between two types of cinnamon i.e. true (*Cinnamomum verum*) and cassia (*Cinnamomum cassia*) cinnamon. For this purpose, we used DNA barcoding genes (*ITS*, *rbcL*, *trnH* and *matK*), random markers like ISSR markers. The ISSR marker, discriminating the two types of cinnamon, was converted into species-specific SCAR marker to be utilized in the authentication and identification of true cinnamon.

Materials And Methods

Plant material and DNA extraction

For the identification and development of species-specific markers, leaf samples were collected from *Cinnamomum verum* and *Cinnamomum cassia*. *Cinnamomum verum* plants were cultivated in the experimental field of CSIR-IHBT, Palampur, and leaf samples of *Cinnamomum cassia* were collected from Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra. Using the conventional CTAB technique, genomic DNA was isolated from the individual leave samples (100 mg). The concentration of isolated DNA samples was quantified using spectrophotometer. Its purity was determined by measuring the OD260/OD280 ratio, and its integrity was checked on 0.8% agarose gel. To make a working solution, all of the stock DNA was diluted to a concentration of 50 ng/μl and stored at -20°C.

Amplification Of Barcoding Gene And Issr Markers

The isolated genomic DNA was amplified using universal barcoding primers for *ITS*, *rbcL*, *matK*, and *trnH-psbA* as well as amplified using 71 different ISSR markers (Table 1). Each reaction for PCR (20µl) consisted of 1X PCR buffer, 0.5 µM of each primer (forward and reverse), 0.5 mM dNTP mix, and 0.5U taq polymerase (GoTaq® DNA polymerase, Promega) and 50 ng of template DNA. The following thermal cycling profile was applied with the BIO-RAD C1000 Touch thermal cycler for amplification-(i) initial denaturation 94°C for 5 min, (ii) 40 cycles of 94°C for 30 s, annealing temperature (55°C for barcoding gene and 50°C for ISSR markers) for 30 s and 72°C for 1 min, and (iii) final extension of 10 minutes at 72°C. All PCR amplified products were resolved on 1.5% agarose stained with 0.5 µg/ml ethidium bromide.

Table 1
Primer sequence used for amplification DNA barcoding genes.

Barcode gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Amplicon Length (bp)
<i>ITS</i>	CCTTATCATTTAGAGGAAGGAG	TCCTCCGCTTATTGATATGC	700
<i>rbcL</i>	ATGTCACCACAAACAGAGACTAAAGC	GTAAAATCAAGTCCACCRCG	650
<i>trnH-psbA</i>	GTTATGCATGAACGTAATGCTC	CGCGCATGGTGGATTCACAATCC	500
<i>matK</i>	GTTCTAGCACAAGAAAGTCG	TAATTTACGATCAATTCATTC	NA
NA = no amplification			

Development Of Sequence Characterized Amplified Region (Scar) Markers

The unique band specific to cassia and true cinnamon was excised and purified from agarose gel using a QIAquick gel extraction kit (Qiagen). The purified product was ligated into pGEMT easy cloning vector (Promega) and transformed into *Escherichia coli* DH5α strained by a heat shock method. Positive clones were selected using blue-white screening on Luria-Bertani (LB) medium plate containing ampicillin (50 µg/ml), Xgal and IPTG. The positive white colonies were confirmed through colony PCR using M13 forward and reverse primer after that confirmed positive clones were isolated using QIAGEN Plasmid mini kit and subsequently the cloned DNA fragments were sequenced through ABI 3130xl DNA Analyzer (Applied Biosystems). Sequencing data was analyzed using FinchTV software to retrieve the nucleotide sequence of the cloned fragments. Based on the sequence information from ISSR sequence, SCAR primer designed using an online tool ApE plasmid. The primer length from 20 bp to 24 bp and the optimum T_m value was adjusted to be ranged of 55°C to 60°C.

The developed SCAR marker was used to amplify cassia and true cinnamon following thermal cycle profile: initial denaturation at 94°C for 4 min, followed by 36 cycles of denaturation at 94°C for 30 secs, annealing at 57°C for 30 secs, extension at 72°C for 1 min and final extension at 72°C for 5 min. PCR products were run on 1.5% agarose gels containing 0.5 µg/mL ethidium bromide.

Result

Amplification and sequencing of barcoding gene

Cinnamomum cassia and *Cinnamomum verum* were amplified with the ITS, *rbcL*, *matK*, and *trnH-psbA* primer (Table 1). Amplification with ITS, *rbcL* and *psbA-trnH* yielded 700bp, 650bp and 500bp respectively, however, *matK* did not show any amplification in both the species (Fig. 1). The PCR amplified product of ITS, *rbcL* and *psbA-trnH* were eluted and sequenced using forward and reverse primers of each gene. After sequencing, quality of each nucleotide was checked and only high quality sequences were used for analysis. Sequence generated by forward primer and reverse complements of sequences generated with reverse primer were aligned to get the final gene sequence. Final gene sequences of *C. cassia* and *C. verum* for individual barcode was aligned using CLUSTALW with following parameters- includes gap open and extension penalty is 6.66 and 15 respectively and IUB weight matrix used for multiple sequence alignment. Surprising, no sequence variation was observed between the cassia and true cinnamon for any of the three barcode genes i.e. ITS (Fig. 2A), *rbcL* (Fig. 2B) and *psbA-trnH* (Fig. 2C).

Screening Of The Issr Markers

In order to develop species specific DNA marker, total of 71 ISSR markers were also screened in *Cinnamomum cassia* and *Cinnamomum verum* for polymorphism (Table 2). These markers resulted in the amplification of total 471 loci. The range of bands produced per primer varied from 2 (ISSR-22) to 17 (ISSR-4) with average 6.63 loci per marker. The size of amplified fragments was ranged from 200 to 2000 bp. Among the analyzed genotypes, 241 of the ISSR fragments were found to be polymorphic (Table 2). ISSR-37 had given a high percentage (100%) of polymorphism (Fig. 3). ISSR-37 produced total found bands in cinnamon, two bands specifically in cassia (746 bp and 500bp) and two bands specifically in true cinnamon (570bp and 350bp). Thus, ISSR-37 clearly distinguished the two cinnamon and was used to develop the SCAR marker.

Table 2

Summary of 71 ISSR markers used for polymorphism screening in *Cinnamomum cassia* and *Cinnamomum verum*.

Marker	Primer sequence (5'-3')	T _m (°C)	Numbers of band*	Numbers of polymorphic bands	% polymorphic bands
ISSR1	TGTGTGTGTGTGTGTGAT	54.2	-	-	-
ISSR2	GAGAGAGAGAGAGAGAC	43.3	-	-	-
ISSR3	GAAGAAGAAGAAGAAGA	45.3	-	-	-
ISSR4	GAGAGAGAGAGAGACC	37.0	17	6	35.3
ISSR5	CTCCTCCTCCTCTC	39.0	9	7	77.8
ISSR6	GACAGACAGACAGACA	45.1	11	3	27.2
ISSR7	CACACACACACACACAGT	53.7	11	9	81.8
ISSR8	CTCTCTCTCTCTCTCTGAC	53.9	9	7	77.8
ISSR9	CACACACACACAAC	35.0	7	3	42.8
ISSR10	CTCTCTCTCTCTCTCTAC	43.4	7	5	71.4
ISSR11	CACACACACACACACAA	52.8	11	3	27.3
ISSR12	CAACAACAACAACAA	44.7	6	4	66.7
ISSR13	AGAGAGAGAGAGAGAGA	47.1	3	1	33.3
ISSR14	CACACACACACACAG	45.0	9	5	55.5
ISSR15	GAGGAGGAGGAGCA	40.0	10	8	80.0
ISSR16	ACACACACACACACACAC	53.5	7	5	71.4
ISSR17	CACCACCACCACCACCAC	66.5	6	2	33.3
ISSR18	GTGTGTGTGTGTGTGTCA	54.9	11	7	63.6
ISSR19	AGAGAGAGAGAGAGC	41.1	8	6	75.0
ISSR20	GAGAGAGAGAGAGAGAA	44.4	13	9	69.2
ISSR21	ACACACACACACACACT	49.3	8	4	50.0
ISSR22	GACAGACAGACAGATC	43.2	2	0	0.0
ISSR23	GTGGTGGTGGTGGTG	57.2	4	2	50.0
ISSR24	AGAGAGAGAGAGAGAGCG	53.6	9	7	77.8
ISSR25	AGAGAGAGAGAGAGAGCA	50.9	8	6	75.0
ISSR26	GAGAGAGAGAGAGAGATG	47.3	8	6	75.0

*"- " represents no clear amplification.

Marker	Primer sequence (5'-3')	Tm (°C)	Numbers of band*	Numbers of polymorphic bands	% polymorphic bands
ISSR27	CTCTCTCTCTCTCTCTAA	44.4	3	1	33.3
ISSR28	AGAGAGAGAGAGAGAGC	46.8	9	5	55.5
ISSR29	AGAGAGAGAGAGAGAGG	46.6	10	6	60.0
ISSR30	ACACACACACACACATC	49.8	8	6	75.0
ISSR31	GAGAGAGAGAGAGAGACTT	48.2	8	6	75.0
ISSR32	GAGAGAGAGAGAGAGACTC	48.5	7	5	71.4
ISSR33	CACACACACACACACAAGG	59.7	9	7	77.8
ISSR34	GTGTGTGTGTGTGTGTCTC	55.3	8	6	75.0
ISSR35	TCTCTCTCTCTCTCTCAGG	53.3	7	5	71.4
ISSR36	ACACACACACACACACCTT	57.1	6	4	66.7
ISSR37	ACACACACACACACACCTA	55.4	4	4	100.0
ISSR38	ACCACCACCACCACCACC	67.2	4	2	50.0
ISSR39	GGAGAGGAGAGGAGA	49.1	6	4	66.7
ISSR40	CGTAGTCGTCACACACACACACA	67.2	8	6	75.0
ISSR41	AGTCGTAGTACACACACACACAC	59.2	-	-	-
ISSR42	ACGACTACGGTGTGTGTTTGTGT	65.5	-	-	-
ISSR43	ATGATGATGATGATGATG	51.3	7	1	14.3
ISSR44	CACACACACACAGG	37.0	8	4	50.0
ISSR45	CACACACACACACACATG	55.6	9	7	77.8
ISSR46	CCGCCGCCGCCGCCG	82.6	-	-	-
ISSR47	CTCCTCCTCCTCCTCCTC	60.5	10	4	40.0
ISSR48	GTGGTGGTGGTGGTGGC	66.7	4	2	50.0
ISSR49	CACACACACACACACAAG	54.6	8	4	50.0
ISSR50	AGAGAGAGAGAGAGAGT	42.5	6	0	0.0
ISSR51	GAGAGAGAGAGAGAGCT	46.8	4	0	0.0
ISSR52	ACACACACACACACACG	54.9	4	0	0.0
ISSR53	AGAGAGAGAGAGAGAGCC	52.2	6	0	0.0
ISSR54	AGAGAGAGAGAGAGAGTA	43.4	7	1	14.3

*"- " represents no clear amplification.

Marker	Primer sequence (5'-3')	T _m (°C)	Numbers of band*	Numbers of polymorphic bands	% polymorphic bands
ISSR55	GAGAGAGAGAGAGAGACT	45.6	6	0	0.0
ISSR56	GAGAGAGAGAGAGAGACG	50.7	8	6	75.0
ISSR57	CACACACACACACACAT	51.2	11	5	45.4
ISSR58	GTGGTGGTGGTGGTGAG	61.2	6	0	0.0
ISSR59	CTCTCTCTCTCTCTCTAG	43.7	6	0	0.0
ISSR60	CGAGAGAGAGAGAGAGA	49.1	5	3	60.0
ISSR61	AGAGAGAGAGAGAGATC	42.9	7	5	71.4
ISSR62	AGAAGAAGAAGAAGACC	47.0	8	4	50.0
ISSR63	ATCATCATCATCATCATCC	55.1	7	3	42.8
ISSR64	ATGATGATGATGATGATGG	56.3	10	2	20.0
ISSR65	CACACAACAACAACAACA	54.2	6	2	33.3
ISSR66	AGCGACGACGACGACGA	67.5	4	2	50.0
ISSR67	AGAACACACACACACAC	48.9	5	1	20.0
ISSR68	AGAGAGAGAGAGAGAGCT	48.8	4	0	0.0
ISSR69	CCACCACCACCACCAGT	63.3	8	2	25.0
ISSR70	AGAGAGAGAGAGAGGCC	47.5	4	0	0.0
ISSR71	GAGAGAGAGAGAGAGACC	49.2	5	1	20.0
*"-" represents no clear amplification.					

Sequence Analyses Of Cloned Fragments And Development Of Scar Marker

Polymorphic DNA fragment of marker ISSR-37 from *Cinnamomum cassia* (746 bp) and *Cinnamomum verum* (570 bp) were eluted and ligated into the pGEMT easy cloning vector. Blue and white colonies were obtained, and white colonies were cultured. The positive cloned were then confirmed by PCR amplification using M13 universal primer. The recombinant clones were selected for sequencing. According to the sequence analysis of cloned fragments, the 746 bp and 570 bp ISSR segments were flanked by the original bases of the microsatellite repeats, as well as no repeats were discovered within the sequence (Fig. 4A and 4B). Further, these sequences were utilized to develop SCAR markers. Single set of primer specific to cassia and verum was designed to produce a stable, reproducible and cassia/true cinnamon specific SCAR markers (Table 3).

Table 3
Sequence characterized amplified region (SCAR) primer developed from ISSR 37 derived amplicon

Species	Cloned sequence length(bp)	SCAR Marker	Primer Sequence	Tm (°C)	Amplicon length (bp)
<i>C. cassia</i>	746	SCAR-CC	Fwd-ACCGGCAACAGGAGAAACGA Rvs-TCCCTCATTAGATCCCTGCATTGA	57.2	312
<i>C. verum</i>	570	SCAR-CV	Fwd-GTTTATGCCCTCTCATTTTGGT Rvs-CAGATTATCTTAGCAGTCAGCAGTC	59.6	190

Amplification Of The Scar Marker

The newly designed SCAR markers were validated by amplifying on sample DNAs from both the species. *Cinnamomum verum* specific SCAR marker were amplified and reproduced a sharp unique band of 190 bp in all the DNA sample of verum and this band is not amplified in the *Cinnamomum cassia* (Fig. 5). However, the SCAR marker specific to cassia was amplified in both the species. One explanation for this is that the SCAR-CC primer sequence might be present at another region in the *C. verum* genome which results in amplification.

Discussion

An essential part of plant product quality control is the accurate identification of plant species, particularly when suppliers may purposefully use the wrong species/cultivars as raw materials (Mulpuri et al. 2013). Nowadays, DNA barcoding is an extremely useful tool, on the basis of a standard region it is functioning as a marker, it has the ability to efficiently and accurately identify species (Li et al. 2021). Marker must fulfill two requirements that are essential for barcoding loci over basic sequence variability that is being universal and being easy to amplify and sequence (Fazekas et al. 2008). In this study, 4 barcode genes (*ITS*, *rbcL*, *matK*, and *trnH-psbA*) were utilized to assess a DNA barcode for the discrimination of true cinnamon and cassia cinnamon. Successful amplification was achieved using *ITS*, *rbcL* and *trnH-psbA* (*matK* genedid not amplify), however, sequencing of amplified products of all the three genes revealed 100% similarity and suggested that these barcoding genes are completely conserved in these two species of cinnamon i. e. *Cinnamomum cassia* and *Cinnamomum verum*. The lack of sequence variation in DNA barcoding genes of *Cinnamomum cassia* and *Cinnamomum verum* might be due to the low rate of sequence evolution between the two spp. For evaluating biodiversity, DNA barcoding is an important tool. However, it frequently fails in complex taxonomy and recently diverged groups, as the Bromeliaceae (Maia et al. 2012). DNA barcode also fail to correctly distinguished species in Hawaiian plant lineages, *Ultricularia* species and Lentibulariaceae (Stallman et al. 2019, Astuti et al. 2020). Additionally, Calligonum species cannot be differentiated through DNA barcoding (Li et al. 2014). Species identification is difficult, especially for closely related species where there is recent diversification, frequent hybridization, and significant intraspecies variation (Li et al. 2014). In plants, repeated DNA sequences are frequently retained at the species or genus level. There are numerous DNA-based markers, including RAPD, AFLP, SSR, and ISSR have the ability to distinguish between species (Joshi et al. 2004). ISSR marker first described by Zietkiewicz (1994) is a

widely used marker approach due to their repeatability, capacity to detect polymorphisms, and cost-effectiveness (Dar et al. 2019). In recent year, ISSR markers have popularly been used to detect polymorphism without the need for sequence information required for primer design, and successfully utilized for species discrimination (Mint Abdelaziz et al. 2020, Raji and Siril, 2021, Bagheri et al. 2022). During the present study, we screened 71 ISSR markers and found that one ISSR marker (ISSR-37) clearly distinguished the two species of cinnamon (Fig. 3). Despite the fact that these DNA molecular markers have several uses for plant DNA analysis, their lack of reproducibility renders them unreliable for direct, quick, and unambiguous identification of plant cultivars. To address these drawbacks, more specific SCAR marker was developed. Because SCAR markers are specific to single loci instead of a complex pattern and their PCR amplification is comparatively insensitive to reaction conditions, they have been widely utilized in the identification of cultivars, species and adulterants (Cai et al. 2022). Based on sequence information, conventional DNA molecular markers like SSR, RAPD, AFLP, SCoT and ISSR can be converted into SCAR markers. This method increases the repeatability of the PCR products of SCAR markers. In the present study, we also converted the cinnamon species (*Cinnamomum cassia* and *Cinnamomum verum*) discriminating ISSR-37 in to the SCAR marker which showed amplification in one species and did not amplify in the other species. In several plant species, SCAR markers were developed from ISSR primer includes ginger (Chavan et al. 2008), *Panax ginseng* (Lee et al. 2011), maple (Boyd et al. 2019), cardamom (Jose et al. 2022) and *Dioscorea polystachya* (Yue et al. 2022). SCAR markers have been employed more often nowadays to verify the authenticity of therapeutic plants such as *Pueraria tuberosa* (Devaiah et al. 2008), *Aconitum heterophyllum* (Seethapathy et al. 2014) and *Dendrobium officinale* (Zheng et al. 2021). Additionally, SCAR markers have been employed to spot adulterants in economically significant plant-derived foods and medicines such as *Crocus sativus* (Torelli et al. 2014), *Punica granatum* (Marieschi et al. 2016) and *Panax ginseng* (Jiang et al. 2018).

The objective of the current work was to identify ISSR polymorphic marker among cinnamon varieties (*Cinnamomum cassia* and *Cinnamomum verum*) to convert them into efficient and more specific SCAR markers. In the present study, a specific ISSR fragment of high intensity and reproducibility was identified for *Cinnamomum cassia* and *Cinnamomum verum* and then was used for development of SCAR primers for consistent and repeatable amplification. A specific 570bp DNA sequence in *Cinnamomum verum* was amplified by ISSR37, which could be used to identify *Cinnamomum verum* samples after being converted into a SCAR marker. In order to maximize the specificity and stability of the amplification product, SCAR marker amplified product was slightly shorter than that of the original particular DNA fragment. The developed SCAR marker (CV) was specific to DNA from all verum samples, as they showed no amplification of DNA from cassia and give distinct 190 bp band in all the verum samples. One new SCAR marker was developed for species identification of *Cinnamomum verum*. It will serve as a useful, rapid and accurate marker for identification as well as helping in authentication.

Declarations

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Authors Contribution

HG- data acquisition, wet-lab experiments, analysis, writing; VG- data acquisition, analysis; RC-

plant material and sampling; SS-Conceptualization, Plant material and sampling, writing and editing, VJ- Conceptualization, Supervision, Data analysis and result interpretation, writing and editing.

Conflict of interest: The authors have no conflicts of interest to declare that are relevant to the content of this article.

Data Availability: All data needed to support the conclusions are included in this article. Additional data related to this paper can be requested from the corresponding author.

Compliance with Ethical Standards

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

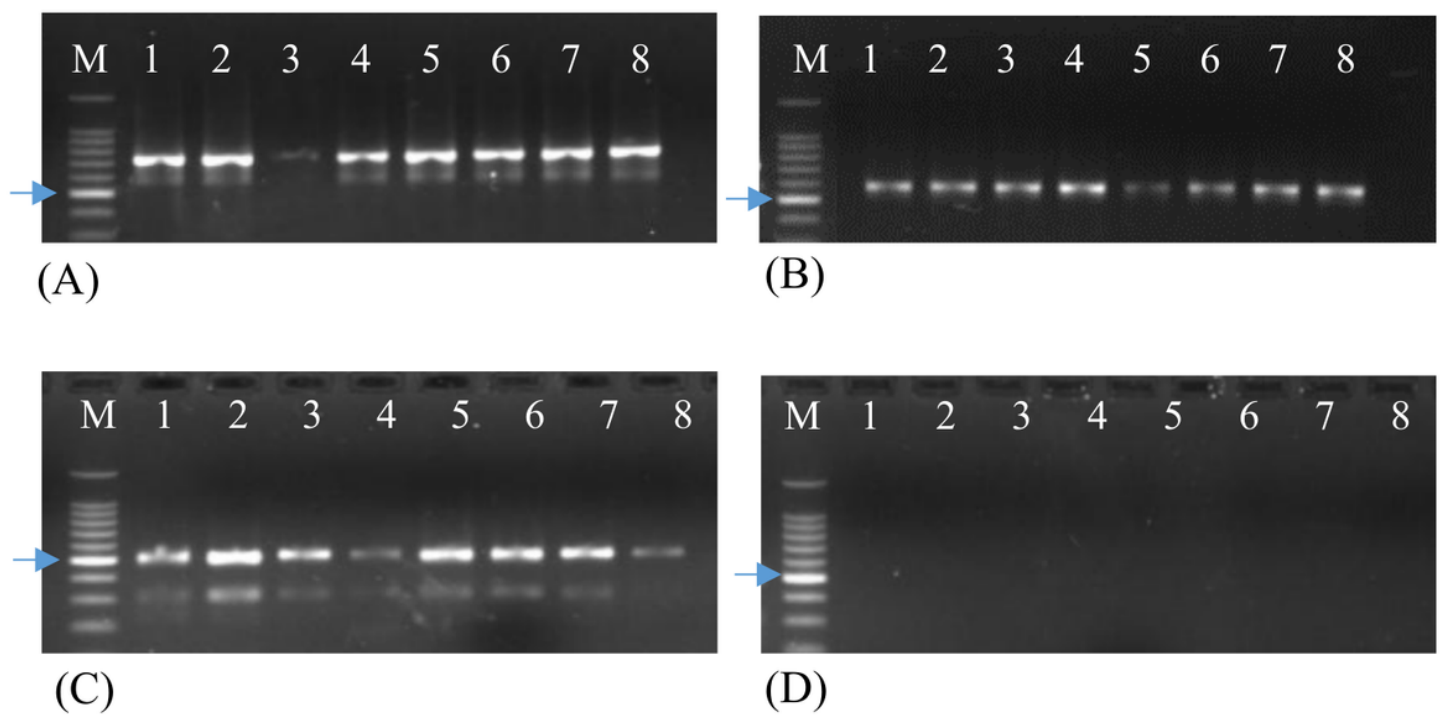


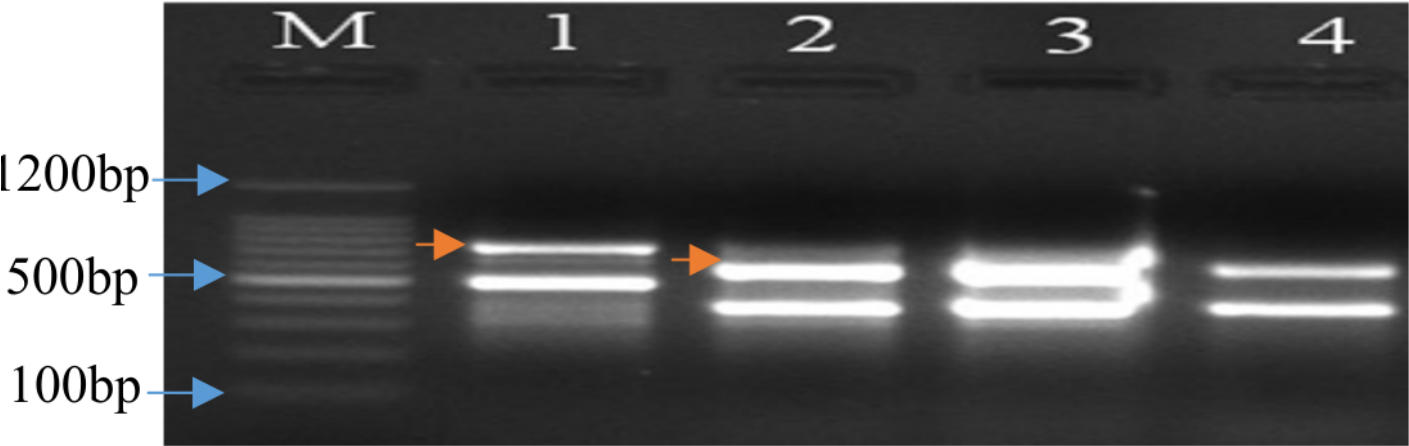
Figure 1

Gel images showing amplification of DNA barcode genes including *ITS* (A), *rbcl* (B), *TrnH-psbA* (C) and *matK* (D) in cinnamon. PCR band with more than 500 bases were amplified in *ITS*, *rbcl* and *TrnH-psbA*, however, no amplification was observed in the *matK* gene. M represents 100 bp DNA ladder, lane number 1-4 and 5-8 resperesnts amplification on *C. cassia* and *C. verum*, respectively. Blue arrow shows 500 bp band.

ITS <i>cassia</i> ITS <i>verum</i>	CCACGGTTGCTAGTGACCTGGGAGGATCATTTGCGATCTGCAATAGCAAGTACCC CCACGGTTGCTAGTGACCTGGGAGGATCATTTGCGATCTGCAATAGCAAGTACCC *****	rbcl <i>cassia</i> rbcl <i>verum</i>	CGTCTATCATGCAGCTGAGCTGTGAAGCTATAAATTGACTTATTACTCTGACTAT CGTCTATCATGCAGCTGAGCTGTGAAGCTATAAATTGACTTATTACTCTGACTAT *****	trnH-psb <i>cassia</i> trnH-psb <i>verum</i>	ACATCCAACTAGCTGCTGTGAGTTCCATCTACAAAGGATAATCTTGGTATTAGTGT ACATCCAACTAGCTGCTGTGAGTTCCATCTACAAAGGATAATCTTGGTATTAGTGT *****
ITS <i>cassia</i> ITS <i>verum</i>	CGCTAATGCTCCCTTGGGCAAGGTATGGGGCTTTGGTCCCAGCAGGAAACCTTG CGCTAATGCTCCCTTGGGCAAGGTATGGGGCTTTGGTCCCAGCAGGAAACCTTG *****	rbcl <i>cassia</i> rbcl <i>verum</i>	GAACCAAGGATACCGAACTTTGGCAGCATTTGAGTAACTCTCACTCGAGTTCGG GAACCAAGGATACCGAACTTTGGCAGCATTTGAGTAACTCTCACTCGAGTTCGG *****	trnH-psb <i>cassia</i> trnH-psb <i>verum</i>	ATACGAGTGTGAAGGATCAATACCAAACTCTTAATAGACAAGAAATTTGGTATTGAT ATACGAGTGTGAAGGATCAATACCAAACTCTTAATAGACAAGAAATTTGGTATTGAT *****
ITS <i>cassia</i> ITS <i>verum</i>	GTAGTGGGCTCTCTCGGGGGCCACTGGCTGCAAAAACCTCGGCGGGAAAGCGCC GTAGTGGGCTCTCTCGGGGGCCACTGGCTGCAAAAACCTCGGCGGGAAAGCGCC *****	rbcl <i>cassia</i> rbcl <i>verum</i>	CCTGAAGAAGCAGGGCGCAGTACTGCCGAATCTTCTACTGGTACATGCAACCTGT CCTGAAGAAGCAGGGCGCAGTACTGCCGAATCTTCTACTGGTACATGCAACCTGT *****	trnH-psb <i>cassia</i> trnH-psb <i>verum</i>	CTATTGGTTCAGTGTGTTTTATCACAATACTGTTTTTCATTTCATTCTTTATTC CTATTGGTTCAGTGTGTTTTATCACAATACTGTTTTTCATTTCATTCTTTATTC *****
ITS <i>cassia</i> ITS <i>verum</i>	AAGGAACCTAAACCTGATTGTACGTCTGATCCCTTATAGGGGCAAGGGGCTTTCCAA AAGGAACCTAAACCTGATTGTACGTCTGATCCCTTATAGGGGCAAGGGGCTTTCCAA *****	rbcl <i>cassia</i> rbcl <i>verum</i>	TGGACCGATGGACTTACGAGCTTTGATCGTTACAAGGGGAGTGTATGGAATGAGGCT TGGACCGATGGACTTACGAGCTTTGATCGTTACAAGGGGAGTGTATGGAATGAGGCT *****	trnH-psb <i>cassia</i> trnH-psb <i>verum</i>	AACCTTAAAGAACCGTGGTATTTTCATGATGAATATGTTCTTCTCTGTACCAAC AACCTTAAAGAACCGTGGTATTTTCATGATGAATATGTTCTTCTCTGTACCAAC *****
ITS <i>cassia</i> ITS <i>verum</i>	AACACACGACTCTTGACACGGATATCTCGCTCTCGCATGATGAAGCCTAGCGAA AACACACGACTCTTGACACGGATATCTCGCTCTCGCATGATGAAGCCTAGCGAA *****	rbcl <i>cassia</i> rbcl <i>verum</i>	GTTCCTGGAGAAGAAAGTCAATTTATTGCTTATGAGCTAACCATTAGACCTTTTGAA GTTCCTGGAGAAGAAAGTCAATTTATTGCTTATGAGCTAACCATTAGACCTTTTGAA *****	trnH-psb <i>cassia</i> trnH-psb <i>verum</i>	TGCATTTTATATCTATTATCTTCAAAATAATTGATTTTTTGAAGAAATCAAGCATT TGCATTTTATATCTATTATCTTCAAAATAATTGATTTTTTGAAGAAATCAAGCATT *****
ITS <i>cassia</i> ITS <i>verum</i>	ATGCGATCTTGGTGTGAATGCGAATCCCGTGAACATCAGTCTTTGAACGCAAGTT ATGCGATCTTGGTGTGAATGCGAATCCCGTGAACATCAGTCTTTGAACGCAAGTT *****	rbcl <i>cassia</i> rbcl <i>verum</i>	GAGGTTCTGTTACTAATCTGTTACTTTTGTAGTAAATGTTGGTTCAAGCCCTG GAGGTTCTGTTACTAATCTGTTACTTTTGTAGTAAATGTTGGTTCAAGCCCTG *****	trnH-psb <i>cassia</i> trnH-psb <i>verum</i>	TTTCTTTTTTTTTTTTTTCAAAATTTTTTTTTTTTAAATTTTTTTTTTCCCGGG TTTCTTTTTTTTTTTTTTCAAAATTTTTTTTTTTTAAATTTTTTTTTTCCCGGG *****
ITS <i>cassia</i> ITS <i>verum</i>	GCGCCGAGGACATTAGCTGAGGCAAGTCTGCTGGGTGTACGCAATTGATTTGCCA GCGCCGAGGACATTAGCTGAGGCAAGTCTGCTGGGTGTACGCAATTGATTTGCCA *****	rbcl <i>cassia</i> rbcl <i>verum</i>	CGTCTCTAAGTCTGGAAGATTGGGAATCCCTACTGCGTATGTTAAACTTCCAGGT CGTCTCTAAGTCTGGAAGATTGGGAATCCCTACTGCGTATGTTAAACTTCCAGGT *****	trnH-psb <i>cassia</i> trnH-psb <i>verum</i>	AGGGAAGGGAAGTAACTAATCAAAAAAGAGAGGGGGAAGGAACCTTTTAA AGGGAAGGGAAGTAACTAATCAAAAAAGAGAGGGGGAAGGAACCTTTTAA *****
ITS <i>cassia</i> ITS <i>verum</i>	CAACACACACTCTTGGAGAGCTGTGCGGTTTGGGGGCGAACTGGGCTCCGCTGCC CAACACACACTCTTGGAGAGCTGTGCGGTTTGGGGGCGAACTGGGCTCCGCTGCC *****	rbcl <i>cassia</i> rbcl <i>verum</i>	CGGCTCAAGGATCCAGTTGAGAGATAAATGAACAAGTATGTCCTCCCTGTTG CGGCTCAAGGATCCAGTTGAGAGATAAATGAACAAGTATGTCCTCCCTGTTG *****	trnH-psb <i>cassia</i> trnH-psb <i>verum</i>	AAAAGGGGGGGGATGTGCCAAGGGATCAGGGCGGGGATTGGAAACCCCCCGGC AAAAGGGGGGGGATGTGCCAAGGGATCAGGGCGGGGATTGGAAACCCCCCGGC *****
ITS <i>cassia</i> ITS <i>verum</i>	TTCTCGGTGTGTGGCAAAAAGCGAGTCTCTGGCTACGAGCTGTGACATTGGTGGTT TTCTCGGTGTGTGGCAAAAAGCGAGTCTCTGGCTACGAGCTGTGACATTGGTGGTT *****	rbcl <i>cassia</i> rbcl <i>verum</i>	GGATGTACTATTAAACCTAAATGGGGTTATCGCTAAAAATACGGTAGAGCTGTTTAT GGATGTACTATTAAACCTAAATGGGGTTATCGCTAAAAATACGGTAGAGCTGTTTAT *****	trnH-psb <i>cassia</i> trnH-psb <i>verum</i>	GG GG *****
ITS <i>cassia</i> ITS <i>verum</i>	GTAAAGAGCCCTCTGCTCTGTTGGCGGATCGGGGTCATCTTAGCGAGCTCAGGACCC GTAAAGAGCCCTCTGCTCTGTTGGCGGATCGGGGTCATCTTAGCGAGCTCAGGACCC *****	rbcl <i>cassia</i> rbcl <i>verum</i>	GAGTGTCTTCCGGGTGATTCGATTTTAC GAGTGTCTTCCGGGTGATTCGATTTTAC *****		
ITS <i>cassia</i> ITS <i>verum</i>	TTAGCGGACACATTTTGTGCGCTTGACGTGACCCAGGTCAGCGGGGACTACACAG TTAGCGGACACATTTTGTGCGCTTGACGTGACCCAGGTCAGCGGGGACTACACAG *****	rbcl <i>cassia</i> rbcl <i>verum</i>			
ITS <i>cassia</i> ITS <i>verum</i>	CTGAGTTTAAACATATCAATAGCGGGAGAA CTGAGTTTAAACATATCAATAGCGGGAGAA *****				

Figure 2

Sequence alignment of barcode genes (A) *ITS* (B) *rbcl* (C) *trnH-psbA* amplified in *C. cassia* and *C. verum*. No sequence variation was observed between the two species for any of three DNA barcodes.



Gel image showing banding patterns of amplification of ISSR-37. M-100bp ladder, 1- *C. cassia* and 2-4 *C. verum*. Size differences in amplicons of the two species are clearly visible. Red arrows are indicating the bands used for development of SCAR marker.

(A)

(B)

DNA sequences amplified with ISSR37 in *C. cassia* (A) and *C. verum* (B). The flanking ISSR37 primer sequences are highlighted in yellow colour.

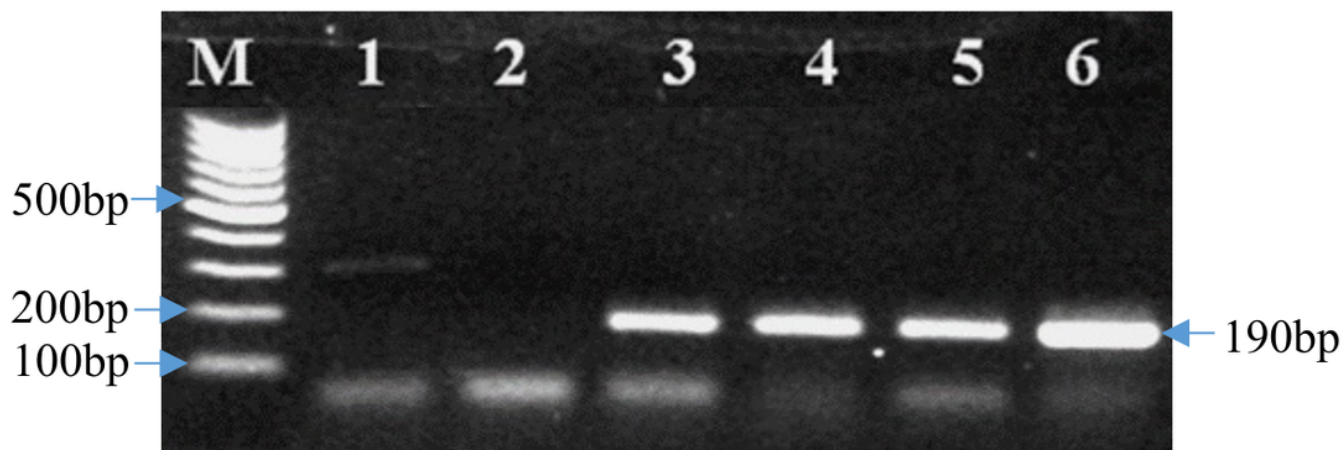


Figure 5

Gel image showing amplification using SCAR-CV marker developed during present study. M-100bp ladder, 1, 2- *C. cassia* and 3-6 *C. verum*. Amplicon of 190 bp of SCAR-CV marker distinguishing the two species.