http://dx.doi.org/10.22161/ijeab/3.5.43 ISSN: 2456-1878

Development of RAPD, DAMD and ISSR markers for authentication of medicinal plant *Cassia auriculata* and its adulterant *Cassia surattensis*

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***Abstract* —** *Cassia auriculata is an important traditional medicinal plant commonly used in many Ayurvedic formulations, meant for diabetes, rheumatism, conjunctivitis, infertility and etc. But due to similar morphological characters and misidentifications, adulteration from Cassia surattensis has been observed. Since safety and efficacy of herbal products has become a major concern due to adulterations, DNA profiling which is an effective and cheap method can be used to solve this problem by discriminating the genuine material. However, no reports about the genetic identification for these species are available to our knowledge. Therefore, the present study was devoted to developing Random Amplified Polymorphic DNA (RAPD), Direct Amplification of Minisatellite-region DNA (DAMD) and Inter Simple Sequence Repeat (ISSR) DNA profiles to authenticate C. auriculata and C. surattensis. As the first step, fresh leaf samples from both plant species were randomly collected from Gampaha district, Sri Lanka and genomic DNA were extracted using modified Cetyltrimethylammonium Bromide (CTAB) protocols. Four short arbitrary primers, two core primers and two SSR primers were used respectively with three different Polymerase Chain Reactions (PCR) based molecular markers which were RAPD, DAMD and ISSR to develop DNA profiles. Out of eight primers, three arbitrary primers, OP-F03, OP-U10 and OP-U20 and one core primer, HBV(5) yielded clear and reproducible amplification products. These results clearly discriminated the medicinal plant C. auriculata and the adulterant C. surattensis providing a complementary tool for quality control of plant derived herbal medicinal products. However, both SSR primers couldn't authenticate two plants and further work is needed to develop ISSR DNA profiles for the authentication.*

***Keywords* — CTAB, DNA profiles, herbal medicines, microsatellites, minisatellites.**

I. INTRODUCTION

Cassia auriculata (Tanners Cassia / Ranawara), is a legume shrub belongs to the large plant family Fabaceae. It prefers drought and dry habitats, therefore can easily found in the tropical climates in India, Sri Lanka and Myanmar [23]. Since it is usually used as a medicine for diabetes, it has a high economic value. Other than in diabetes, *C. auriculata* is widely used in traditional medicine for rheumatism, conjunctivitis, diarrhea, female infertility, leprosy and also for skin diseases [17].

Species such as *Cassia surettensis* and *Cassia divericata* are morphologically indistinguishable from *C. auriculata*, since flowers of these species are more or less similar in morphology to each other and therefore, easily adulterated specially during harvesting. And also adulterations can arise due to the price pressure, increased demand, limited availability, deceitful substitution using less valuable species and indistinct taxonomy due to confusion between Latin nomenclature and local terminology. Importantly, adulterants do not have medicinal values as in *C. auriculata* and some contains toxic compounds [04]. Therefore, an accurate, effective and reliable method is essential for the authentication of the *C. auriculata* plant to avoid adverse effects on herbal medicine consumers.

Morphological based authentication of medicinal plants may not provide the correct identification due to similarities in morphology between original plant and adulterants, and also due to geographical variations in plants of same species. Chemical based methods cannot also use due to variations in the chemical composition arising from age and geographical variations.

Since the genetic composition is unique to each species and it is not affected by age, physiological and geographical factors, DNA based molecular authentication is the most desirable way to authenticate those medicinal

http://dx.doi.org/10.22161/ijeab/3.5.43 *ISSN: 2456-1878*

plants. Therefore, DNA markers and PCR based DNA profiling techniques can be used [02].

DNA profiling is a technique which is used to identify an individual from a DNA sample by looking at the unique DNA banding patterns which result from unique DNA markers after PCR and gel electrophoresis [07]. It can be done in two ways. Those are PCR based single-locus method and PCR based multi-locus method. Among them multi-locus method is mostly and efficiently used with genomes where genome sequence data are scantly or lacking. Multi-locus method uses single oligonucleotide primers with universal arbitrary sequences to produce DNA markers from genomic DNA resulting in multi-locus banding patterns after gel electrophoresis. RAPD, ISSR and DAMD PCR profiling methods are popular variants of this method [13].

In RAPD-PCR profiling, short arbitrary random synthetic oligonucleotide primers are used to amplify multi locus DNA markers which have distributed within the genome [24]. In ISSR-PCR method, DNA segment present in between two identical Simple Sequence Repeat (SSR) regions (Microsatellite regions) oriented in opposite directions is amplified [14] using SSR regions as primers. These primers target multiple loci within the genome to amplify Inter Simple Sequence Repeats (ISSRs) of different sizes, producing multi locus dominant markers. Most of the minisatellite sequences which have dispersed among many organisms share common central motifs which are known as core sequences or core units. These core sequences can be used to design universal primers to use in DAMD-PCR profiling of previously unknown genomes. Core sequence of minisatellite regions is used as a single primer and it can anneal with two adjacent core sequences which have located in opposite directions within two minisatellite regions. Therefore, amplified PCR products are rich in minisatellite repeats and show polymorphism due to site specific length variation in the inter repeat region as in ISSR-PCR method [16, 24].

C. auriculata and its adulterants do not have well defined genomes and therefore, this study was undertaken to authenticate *C. auriculata* plant from its adulterant *C. surattensis* plant using RAPD, ISSR and DAMD DNA profiling.

II. MATERIALS AND METHODS

2.1 Sample Collection

Fresh, young and healthy leaf samples of *C. auriculata* and *C. surattensis* were randomly collected respectively from five and three locations in Gampaha district, Sri Lanka. Plants were identified by Dr. (Ms.) T. D. Ramanayake (DAMS). Collected leaf samples were washed well with tap water and surface sterilized with 70% Ethanol followed by three serial washings with sterilized distilled water. Then leaves were allowed to air dry on sterilized filter papers. After drying, midribs of leaves were removed using a sterilized scissor and remaining leaf parts were measured using analytical balance (CY 720-Citizen Scale, USA) as portions of 1.00 g. Each portion was covered with an aluminium foil and stored at -80 °C until further use.

2.2 DNA extraction

Plant genomic DNA were extracted from all samples according to the protocol described by Sahu *et al.* (2012) after several modifications.

After all modification events two different CTAB extraction buffers have to use with two plant species to obtain highly purified genomic DNA. CTAB extraction buffer consisting 2% (w/v) CTAB, 100 mM Tris HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 3% (w/v) PVP and 2% β-mercaptoethanol was used with *C. auriculata* plant samples and buffer containing 2% (w/v) CTAB, 100 mM Tris HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 4% (w/v) PVP and 2% β-mercaptoethanol was used with *C. surattensis* leaf samples. Both CTAB extraction buffers were preheated at 65 °C in a water bath for 30 minutes. Frozen leaf samples (1.00 g of each) were crushed using ice cold mortar and pestle. Finely ground tissues were added into microfuge tube (2 mL) which contained relevant pre heated extraction buffer (1.00 mL). Then they were mixed vigorously and incubated at 65 °C for 30 minutes in a water bath with occasional swirling. After incubation tubes were cooled to room temperature and centrifuged (Hettich Mikro 200 Zentrifugen, Germany) at 13,000 rpm for 5 minutes and the supernatants were transferred into new microfuge tubes. Equal volumes of chloroform: isoamyl alcohol (24:1) were added and mixed gently by inverting 5 minutes. Then the tubes were centrifuged at 13,000 rpm for 2 minutes and the supernatants were collected to new microfuge tubes. Re-extraction with chloroform: isoamyl alcohol (24:1) was done and the aqueous supernatants were transferred to new microfuge tubes. Ammonium acetate (7.5 M, 100 µL) followed by ice cold absolute alcohol (1.00 mL) was added to the transferred supernatants and tubes were gently inverted few times. Then the tubes were incubated at -80 °C for 2 hours. After that tubes were centrifuged at 13,000 rpm for 5 minutes to precipitate the DNA and then the supernatant was removed. The DNA pellet was washed with 1.00 mL of Ethanol (95%) and was agitated vigorously to release the pellet. Then tubes were centrifuged at 13,000 rpm for 5 minutes to pellet out the DNA again. Then the supernatant was removed and microfuge tubes were air dried on filter paper for 10 minutes. Then, the DNA pellet was resuspended in 50–100 µL of TE buffer. Then RNase A (2.00 µL, 10µg/ml) was added and incubated at 37 °C in dry bath for 1.5 hour. Finally, extracted DNA samples were stored at -20 °C

until further use. DNA concentration and purity were determined by measuring the absorbance of 25 times diluted DNA solution at 230 nm, 260 nm and 280 nm respectively using ORION Aquamate 8000 UV-VIS spectrophotometer. The quality of the genomic DNA was checked on 0.8% agarose gel stained with Ethidium Bromide.

2.3 PCR reactions

Eight primers which can be used in PCR amplifications were selected according to the research that Ranade and Farooqui has conducted to authenticate medicinal plant Neem (*Azadirachta indica*) in year 2002 (Table 1).

Table.1: The microsatellites, minisatellites and arbitrary sequences used as primers in PCR amplification reactions.

No.	Primer name	Primer sequence (5'- 3')
1	TATC-6	TAT CTA TCT ATC TAT CTA TCT ATC
2	GATA-6	GAT AGA TAG ATA GAT AGA TAG ATA G
3	33.6	GGA GGT TTT CA
4	HBV(5)	GGT GTA GAG AGA GGG GT
5	OP-F02	GAG GAT CCC T
6	OP-F03	CCT GAT CAC A
7	OP-U10	ACC TCG GCA C
8	OP-U20	ACA GCC CCC A

2.3.1 RAPD-PCR

Four arbitrary primers of OPF and OPU series were used in RAPD analysis. The reactions were carried out in 25 µL volume in a tube using four RAPD primers which were OP-F02, OP-F03, OP-U10 and OP-U20. Each reaction tube contained 0.5 µL template DNA, 1× GoTaq® Flexi Buffer, 2.5 mM $MgCl_2$, 200 µM of dNTPs, 0.4 µM of primer and 0.5 units of GoTaq® DNA polymerase. Amplification reaction was performed in a thermal cycler (Veriti® 96-Well Thermal Cycler, Applied Biosystems, USA), using following conditions: pre denaturation at 94 ºC for 2 minutes followed by 45 cycles of denaturation at 94 ºC for 30 s, annealing at 35 ºC for 30 s and extension at 72 ºC for 1 minute and final extension at 72 ºC for 7 minutes.

2.3.2 ISSR-PCR

Two SSR primers, TATC-6 and GATA-6 were used. Pilot experiments using a range of annealing temperatures were performed to optimize the annealing temperature for each SSR primer, along with different DNA volumes. The annealing temperature range was determined in the range of 2-10 °C lower than the denaturation temperature (Td) of each primer. As mentioned by Ranade and Farooqui (2002), the Td was calculated by adding 2 °C for each A or T and 4 °C for each G or C in the primer sequence. According to that calculation, the Td of primer TATC-6 is 60 °C and Td of GATA-6 primer is 64 °C. Therefore a temperature gradient PCR which includes 52 °C, 54 °C, 55 °C, 57 °C and 58 °C combined with a range of DNA volumes (0.50 µL, 1.00 µL, 2.00 µL and 5.00 µL) was carried out with primer TATC-6. Another temperature gradient PCR which includes 54 °C, 55 °C, 57 °C, 60 °C and 62 °C combined with the same range of DNA volumes as in the above reaction was used with primer GATA-6. Other reagents in the reaction mixture (25 µL final volume) were 1× GoTaq® Flexi buffer, 3.0 mM $MgCl_2$, 200 µM of dNTPs, 0.4 µM primer and 0.5 units of GoTaq® DNA polymerase. PCR cycles were carried out with pre denaturation at 94 ºC for 5 minutes followed by 25 cycles of denaturation at 94 ºC for 30 seconds and extension at 72 ºC for 1 minute and final extension at 72 ºC for 7 minutes in the thermal cycler.

2.3.3 DAMD-PCR

Two DAMD primers named HBV(5) and 33.6 were used for DAMD analysis. The PCR reaction mixture of 25 µL contained 5.00 µL of genomic DNA, 1× GoTaq® Flexi Buffer, 3.0 mM $MgCl_2$, 200 µM of dNTPs, 0.4 µM of primer and 0.5 units of GoTaq® DNA polymerase. During the amplification reaction in the thermal cycler, pre denaturation step of 5 minutes at 94 ºC was followed by 35 PCR cycles (denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 1 minute, extension at 72 ºC for 1 minute). A final step of 7 minutes at 72 ºC was carried out for the polishing of PCR products.

In all PCR amplification reactions a sample without template DNA was used as a negative control to check the presence of contaminations.

2.4 Gel electrophoresis

The reaction products obtained after PCR were electrophoresed on 1.5% agarose gel stained using ethidium bromide in 1× TBE buffer at 50 V for 1.5 hours using a horizontal electrophoresis apparatus (APPLEX PS 9009 TX, France). Electrophoretic profile was visualized under UV transilluminator and documented through a Gel Documentation System (QUANTUM ST5, Germany). The sizes of DNA fragments were estimated by comparison with a 100 bp or 1 kb molecular weight marker (Promega Corporation).

http://dx.doi.org/10.22161/ijeab/3.5.43 *ISSN: 2456-1878*

III. RESULTS AND DISCUSSION

"Herbal drugs" is a main part of the traditional medicine especially in developing countries. According to surveys, about 75% of the world population depends on traditional medicine [02]. The traditional medicine system basically depends on large number of medicinal plants, which represents a huge biodiversity. Rapid and accurate authentication of medicinal plants is difficult to obtain, at the scale of international trade. Especially because, many medicinal plant based commercial products are sold either in dried form or as processed material, leading to their authentication by morphological methods very difficult or impossible. But, DNA based methods such as DNA-profiling can be used to identify adulterated raw materials more accurately and effectively. PCR based DNA-profiling method has been successfully used as a molecular authentication system of several medicinal plants. Neem [16], Oregano [11], Saffron [06] and *Acorus calamus* [15] are some successful examples.

In this research, a medicinal plant *Cassia auriculata* was chosen for the authentication through PCR based DNA-profiling. To the best of our knowledge, this is the first DNA profiling based molecular authentication experiment which has done with *C. auriculata*.

Cassia surattensis can be found as adulterant in *C. auriculata* based herbal medicinal products which is available in the market. *Cassia divericata* also can be used as an adulterant, but it is a rare plant in Sri Lanka, therefore it is impossible to use it as an adulterant in the market samples. Due to these reasons, in this study *C. surattensis* plant samples were also used for differential identification.

When moving steps into molecular authentication, the initial step which have to do is DNA extraction. Molecular authentication is a downstream application of DNA extraction. Therefore, high quality and highly purified genomic DNA free from proteins, RNA and other secondary metabolites such as polyphenols, polysaccharides and terpenes should be isolated from both *C. auriculata* and *C. surattensis* species to obtain successful final result from molecular authentication. Generally OD260/280 ratio of pure dsDNA should be around 1.8 and OD260/230 ratio should be between 2.0 and 2.2 [03]. It means that, OD ratio values which obtained other than these standard values indicate the presence of contaminations in extracted genomic DNA. When extracting genomic DNA from *C. auriculata* leaf samples, optimization of DNA extraction protocol was essential, since initial DNA extraction protocol [18] used in this study ended up with low quality DNA with good yield from 1.00 g of fresh leaf sample. After the modifications in the initial DNA extraction protocol extracted genomic DNA had a 1.82 ± 0.03 of OD260/280 ratio and a 1.85 ±0.07 of OD260/230 ratio with a yield of 740.00 ± 47.55 µg/mL. According to Semagan (2013), OD260/230 is a secondary measure of nucleic acid purity and therefore that ratio of 1.8–2.2 is acceptable for downstream applications such as PCR. Therefore DNA extractions from *C. auriculata* leaf samples collected from all five locations were carried out after the modifications of CTAB protocol.

Then, this optimized CTAB DNA extraction protocol was used to extract genomic DNA from adulterant *C. surattensis* leaves. Extracted genomic DNA resulted in low OD ratio values while providing good yield. Therefore, modifications were needed with this extraction also. After several optimization events, genomic DNA with OD260/280 ratio of 1.85 ± 0.04 and OD260/230 ratio of 2.00 ± 0.20 was obtained with 1085.83 ± 492.38 µg/mL yield. Both obtained values were in the standard OD ratios. Therefore, DNA extraction from *C. surattensis* leaf samples collected from all three locations were carried out after the modifications in CTAB protocol.

The next step which have to do is molecular authentication of those two plant species using extracted high quality DNA. In that case DAMD-PCR profiling, RAPD-PCR profiling and ISSR-PCR profiling were used.

DAMD-PCR technique was first described by Heath *et al.* (1993) and has been applied with various plant species including *Triticum* [01] and *Capsicum* [09]. In this PCR technique, a single primer from a minisatellite core sequence is used to direct PCR by amplifying regions rich in minisatellites. These regions may have core sequences involved in inversions between successive minisatellites on both strands in opposite orientations, allowing the primer annealing and amplification of minisatellite regions [24]. HBV(5) [12] and 33.6 [10] are famous core sequences which have been used as DAMD primers. *Homo sapiens* (Human) genome is the source of these primers, but they are conserved across many other species making them as universal primers [12]. Therefore, when conducting DAMD-PCR reaction in this study, these two primers were used. For any PCR, it was necessary to standardize the genomic DNA concentration which need to contain in the PCR mixture, since higher DNA amounts can inhibit PCR reaction and lower DNA concentrations cannot produce sufficient amount of amplicons to be visualized. Therefore, a pilot experiment was conducted with a series of genomic DNA volumes from DNA stocks as 1.00 µL, 2.00 µL and 5.00 µL to check the best amplification. All DNA dilutions provided DNA profiles with primer HBV(5), but the best profiles with clearly visible bands were obtained with 5.00 µL genomic DNA volume in the PCR mixture (Fig. 1).

But, a profile with faint bands was observed with the Sample no. 7. It can be due to many practical errors. However, the amplification products of profiles were in the range of 450–1250 bp and those bands had an equal

http://dx.doi.org/10.22161/ijeab/3.5.43 *ISSN: 2456-1878*

degree of polymorphism and stability. But the scoring of those markers was difficult due to low separation. According to Ince *et al.* (2008), this problem could be reduced by using Agarose gels such as 25–30 cm in length with a long running time. With the primer 33.6 no profiles were obtained even with any genomic DNA volume. It may be due to the absence of that particular core sequence in minisatellite regions within the genomes of these two species.

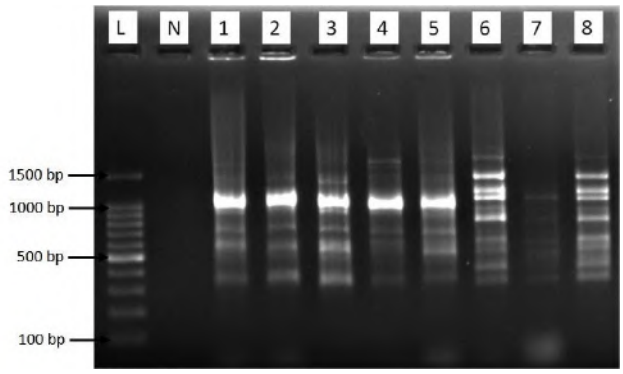

Fig. 1: DAMD-PCR profiles of C. auriculata and C. surattensis for primer HBV(5). Starting from the left, Lanes: L, 100 bp DNA ladder; N, negative control; 1-5, C. auriculata; 6-8, C. surattensis. The lane numbers mentioned correspond to the samples collected from different locations.

RAPD-PCR technique was first described by Williams *et al.* in the year 1990. Thereafter, this technique has been successfully used with many plant species including different medicinal plants such as *Panax* [20] and *Aloe* [21]. In this technique DNA polymorphism is determined based on the amplification of random DNA segments in the genome using single primers of arbitrary nucleotide sequences. In this study, four arbitrary primers OP-F02, OP-F03, OP-U10 and OP-U20 were used. In these reactions also, it was necessary to standardize the genomic DNA concentration which has to contain in the PCR mixture. Therefore, a pilot experiment was conducted with a series of genomic DNA volumes from DNA stocks as 0.50 µL, 1.00 µL and 2.00 µL to check the best amplification. After gel electrophoresis, smears were observed with 1.00 and 2.00 µL DNA volumes and it can be due to high concentrations of DNA. But with 0.50 µL genomic DNA volume, good banding patterns were observed with all four primers. The amplification products of those profiles were in the range of 350–1600, 350–2000, 450–1000 and 350–1600 with primer OP-F02 (Fig. 2. A), OP-F03 (Fig. 2. B), OP-U10 (Fig. 3. A) and OP-U20 (Fig. 3. B), respectively. Profiles with clear and well distinct bands were observed with three primers other than the primer OP-F02. The profile obtained with primer OP-F02 contained faint bands, with some plant samples such as Sample no. 2, 3, 4, 5 and 6. It can be due to the low reproducibility of that particular primer OP-F02. Due to low reproducibility, other three RAPD profiles obtained from this experiment are more suitable to differentially authenticate these two plant species successfully.

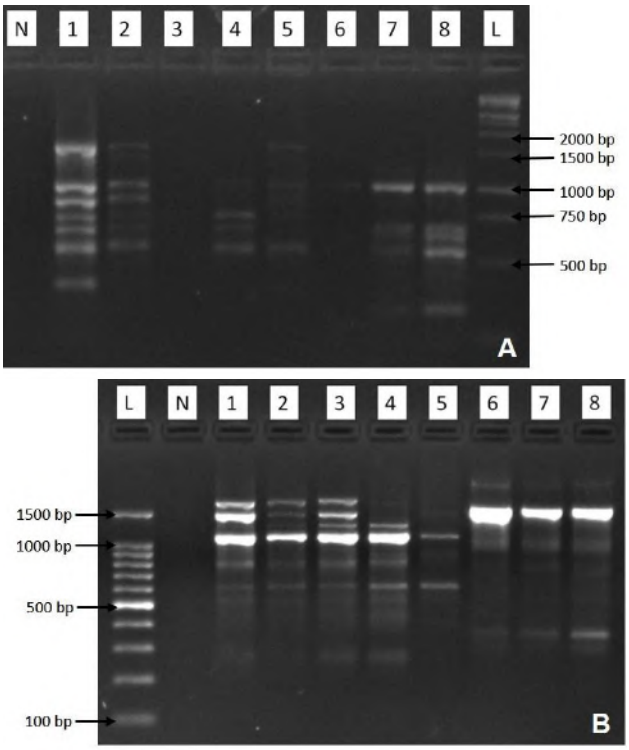

Fig. 2: RAPD-PCR profiles of C. auriculata and C. surattensis for ***A,*** *primer OP-F02 and* ***B,*** *primer OP-F03. Starting from the left, Lanes: L, 100 bp/1 kb DNA ladder; N, negative control; 1-5, C. auriculata; 6-8, C. surattensis. The lane numbers mentioned correspond to the samples collected from different locations.*

ISSR-PCR technique also has been successfully used to identify some plant species including *Citrus* cultivars [05], *Pseudotsuga menziesii* and *Cryptomeria japonica* [22]. ISSR-PCR involves a single primer composed of microsatellite / SSR sequence. This primer can target microsatellite regions and amplify the intervening region between the two microsatellite regions in opposite orientations [07]. In this research, two ISSR primers have been used which are TATC-6 and GATA-6 but, there were no standardized annealing temperatures for these primers. Therefore, pilot experiments using a range of annealing temperatures were performed to optimize the annealing temperature for each SSR primer along with a range of template DNA volumes to determine the optimum DNA volume which should be in the PCR mixture. But, no profiles were observed for any SSR primer. It can be due to the lack of these primer sequences within the *C. auriculata* and *C. surattensis* plant genomes. Also according to Ranade and Farooqui (2002) most of the SSR

http://dx.doi.org/10.22161/ijeab/3.5.43 *ISSN: 2456-1878*

primers with tetra-nucleotide repeats could not result in any amplification only result in a smear or no profile as in this study and tri-nucleotide SSR primers were more effective and resulted in clear distinct amplification profiles. This can be due to the low annealing ability of these primers. Therefore, tri-nucleotide SSR primers such as CAC-5 and GAA-6 which have succeeded in PCR-profiling of medicinal plant Neem as mentioned by Ranade and Farooqui (2002), may be successful with these two plant species also.

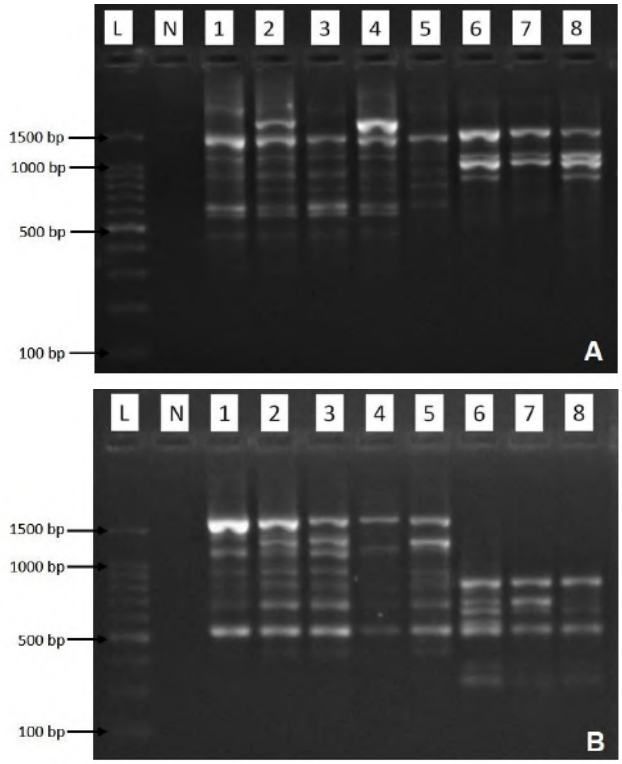

*Fig. 3: RAPD-PCR profiles of C. auriculata and C. surattensis for **A,** primer OP-U10 and **B,** primer OP-U20. Starting from the left, Lanes: L, 100 bp DNA ladder; N, negative control; 1-5, C. auriculata; 6-8, C. surattensis. The lane numbers mentioned correspond to the samples collected from different locations.*

According to this study, though RAPD-PCR profile with primer OP-F02 is not suitable and ISSR-PCR profiling cannot be used to authenticate *C. auriculata* and *C. surattensis* plants effectively, other four DNA profiles which obtained with primer OP-F03, OP-U10, OP-U20 and HBV(5) can be used to differentially identify *C. auriculata* and *C. surattensis* plants effectively. Unique DNA banding patterns specific to each *Cassia* species with different primers have summarized in Table 2. Those unique banding patterns can be either used to authenticate two plant species or those plant derived herbal medicinal products which is available in the market.

Table.2: Species specific bands (bp) obtained with five different primers in PCR amplifications.

Primer	Species specific bands (bp)	
	Cassia auriculata	***Cassia surattensis***
HBV(5)	-	1250
	-	1100
	1000	-
	750	-
	-	650
	-	450
OP-F03	-	2000
	1650	-
	1100	-
	-	1000
	800	-
	600	-
	-	350
OP-U10	-	1000
	900	-
	-	800
	750	-
	600	-
	550	-
	450	-
OP-U20	1600	-
	1250	-
	950	-
	-	720
	700	-
	-	350

IV. CONCLUSIONS

To the best of our knowledge this is the first attempt of the genomic based authentication of medicinal plant *C. auriculata* and its adulterant *C. surattensis*. RAPD and DAMD PCR markers developed in this study provide guidance to authenticate two plant species effectively and reliably. Nonetheless, further studies are required to introduce another SSR primer to develop ISSR PCR markers for this authentication.

ACKNOWLEDGMENT

This work was supported by the department of Botany, University of Kelaniya, Sri Lanka. And authors would like to acknowledge Prof. Piyal Marasinghe and Dr. T. D. Ramanayake for their constructive suggestions.

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