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Original Article

SCAR MARKER DEVELOPMENT FOR THE CORRECT IDENTIFICATION OF *IRIS ENSATA*

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ABSTRACT

Objective: The objective of this research was to develop the RAPD based SCAR marker for the correct identification of the *Iris ensata* Thunb. (*I. ensata*) plant from its adulterants.

Methods: Five samples of *I. ensata*. from the different geographical area were used in this study. The plant genomic DNA was isolated with the CTAB method with some modification (as dried samples were also used). After that, polymorphism was checked with the help of the 10-mer random primers of OPAA and BG series. Then, the bands of interest were eluted and cloned into pGEMT easy vector for the sequencing. Finally, the sequence is used to develop the SCAR primers (Ir-f andIr-R) specific for *I. ensata* and the developed primers also validated with respect to the market samples.

Results: A putative 580 bp sequence specific for *Iris ensata* was identified from the randomly amplified polymorphic DNA (RAPD) analysis. To overcome the main limitation of RAPD it has been converted into SCAR markers. So that, this specific band was then eluted, cloned and sequenced. After that, SCAR primers (Ir-F and Ir-R) were synthesized by using this sequence. For the validation of the synthesized SCAR primers, they were tested with respect to the market samples. The amplicon of 260 bp was produced by the SCAR primers in the authentic *I. ensata* but market samples did not produce any bands with the synthesized SCAR primers.

Conclusion: The results of this study show a high level of polymorphism in the RAPD pattern of the different accessions of the plant. Furthermore, this study results in the successful development of the RAPD based SCAR marker for the identification of the *I. ensata*.

Keywords: Herbal, *Iris,* Medicinal plant, RAPD, SCAR

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INTRODUCTION

In *Iridaceae* family, *Iris* is the major genus which has near about 230 species [1]. It provides models for the introgressive hybridization and hybrid fitness studies of the plant [2]. The plant is mainly found in China, Japan, Korea, and Russia [1]. The *I. ensata* species of *Iris* is one of the generally cultured, crossbred, and imperative horticultural species [3] and has several essential conjoint cultivars recognized as 'Japanese *Iris.*' 'Hanashobu' (Japanese common name).

The roots of the plant have many important medicinal properties like it is used to kill parasitic worms, used in hepatic diseases, used as an appetizer, as a diuretic agent, as a detoxifying agent and as an antidote [4-6]. This plant is also used in combination with other plants to treat various diseases such as liver grievances, dropsy and venereal affections [7].

Molecular biology provides various techniques that can be useful for plant identification from their adulterant [8]. At the genetic level, polymorphism has been broadly studied in medicinal plants which help in distinguishing plants at inter or intraspecies level [9-10]. DNA-based molecular markers can be used in the enhancement of medicinal plant species. These markers can be used for evaluating genetic diversity in plants, authenticating plant material from their adulterants thereby utilizing them for MAS breeding and therapeutics. From the last two decades, the PCR (Polymerase Chain Reaction) became one of the main methods for the nucleic acid analyses [11]. PCR based markers including Randomly Amplified Polymorphic DNA (RAPD) can be proficiently used for verification of the medicinal plant material [12].

Furthermore, RAPD marker also has been used to study the genetic variations among the geologically different population of fungi [13]. In herbal medicine, RAPD analysis has been used to differentiate between species in numerous genera [14]. The necessity of small amounts of plant material, quickness and low cost are the advantages of RAPD [14-15]. RAPD reveals a high degree of polymorphism without the requirement of prior DNA sequence information and it is simple to perform. The RAPD technique amplified unique fragments as this technique does not amplify two discrete fragments that co-migrate on gels having a similar size [16]. Therefore, this application is explored by various researchers for authentication of traditional medicinal plants [17]. Reproducibility of the RAPD results is low because this technique is sensitive to PCR conditions. To prevail over this problem, RAPD has to be converted into sequence characterized amplified region (SCAR) marker [18]. Further, industrial application of the molecular techniques can be increased by the development of more specific, sensitive and reproducible markers like Sequence Characterized Amplified Region (SCAR). In the present study, we developed reliable SCAR markers based on the amplified product of the RAPD primers for the identification of *Iris ensata* from its adulterants.

MATERIALS AND METHODS

Plant material

Five different accessions of *I. ensata* were used in this study. Roots and aerial parts of five different accessions were collected from different geographical areas of India (table 1). These samples were identified by Prof. M. P. Sharma, Head, Dept. of Botany, Jamia Hamdard, voucher specimen is deposited at the herbarium (No. DM/JH/251-a/2013-1, DM/JH/251-b/2013-14, DM/JH/251-c/2013-14, DM/JH/251-d/2013-14, DM/JH/251-e/2013-14) of Department of Botany, Jamia Hamdard, New Delhi. For the validation of marker two market samples of the plant were also collected from the local herb market of Khari Baoli, Delhi, India.

Chemicals

All chemicals used in the study were molecular grade. EDTA (Ethylenediaminetetraacetic acid), CTAB (Cetyl Trimethyl

Ammonium Bromide), NaCl, Tris HCL, Agarose, β-mercaptoethanol, Phenol, Chloroform, Isoamyl alcohol, Ammonium acetate were purchased from Hi-media, Mumbai, India. The RAPD primers, PCR reaction mixture, and RNase were purchased from Merck (Darmstadt, Germany), whereas the developed primers (SCAR) were purchased from IDT, USA.

Table 1: List of plant materials (*I. ensata*) used in this study

Plant name	Plant part	Sample	Locality
I. ensata	Aerial	Ir-1	IHBT, Palampur
	Aerial	Ir-2	University of Kashmir, Srinagar
	Root	Ir-3	Chopta forest, Himalayas
	Root	Ir-4	Chamba, Himachal Pradesh
	Root	Ir-5	Hamdard laboratory, Ghaziabad

Genomic DNA isolation

Sample preparation

Dried root samples of *Iris ensata* were chopped into small pieces and kept overnight in distilled water to make them soft then these roots samples were pressed to remove excess water and these soft roots were used for genomic DNA isolation.

DNA extraction

Genomic DNA of root sample of *I. ensata* was isolated by CTAB extraction method [19] with some modifications. 4.0 g of root sample was ground in liquid nitrogen to a fine powder. 15 ml of pre-warmed (65 °C) 4 x CTAB buffer [4% CTAB, 1.4 M NaCl, 0.5 M EDTA (18.61 gm/100 ml) pH-8, 0.5 M Tris-HCl (15.76 gm/100 ml) pH-8] and 0.2% β-mercaptoethanol was added just before use. The homogenate was incubated at 65 °C in a water bath for 45 min with intermittent mixing. An equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and mixed well by inverting the tubes 3-4 times. The suspension was then centrifuged twice at 10000 rpm for 15 min at 25 °C. The aqueous phase was transferred to a fresh tube and 5 µL of RNase (50 mg/ml) was added to the aqueous phase and incubated at 37 °C for 1 hour. The aqueous phase was extracted again by adding an equal volume of Chloroform/Iso amyl alcohol (24:1). The suspension was mixed well and then centrifuged for 15 min at 4 °C at 10000 rpm. The aqueous phase was again transferred to a fresh tube. One volume of chilled Isopropanol was added to the aqueous phase and incubated at-20 °C for overnight for precipitation of DNA. The DNA was pelleted by centrifugation at 10000 rpm for 15 min at 4 °C. DNA pellet was then washed with washing solution (70 % ethanol, 2M ammonium acetate) and air dried. The pellet was dissolved in an appropriate volume (200-300 µL) of TE buffer or sterile Milli-Q water. The DNA solution was stored at 4 °C for immediate use or at-20 °C for prolonged storage.

RAPD analysis

A set of 40 random primers of OPAA and BG series was used for the PCR screening of genomic DNA of all five accessions of *I. ensata* (table 2). Ten primers showing clear and reproducible polymorphic patterns in preliminary trials were selected to analyze the further RAPD. PCR reactions were carried out in the 15 µl reaction volume containing (5U µl) Taq polymerase, 1x *Taq* buffer (10 mmol), $MgCl_2$ (10 mmol), dNTPs (2.5 mmol each), 10 µM of each primer and 50 ng/µl of DNA template. Amplification was performed in a thermal cycler (T100™ Thermal Cycler, Bio-Rad, USA).

PCR conditions were set as: initial denaturation 95 °C for 4 min, denaturation at 94 °C for 30 sec, annealing at 32 °C for 45 sec, extension at 72 °C for 50 sec, and a final extension at 72 °C for 2 min and infinite hold on 4 °C. Amplified PCR products (15 µl) along with 3 µl 6X bromophenol dye were loaded on 1-1.2% agarose gel containing ethidium bromide (0.5 µg/ml) prepared in 1X TAE buffer and electrophoresed on 100 volts for one and half hour. The amplified DNA was visualized on UV lamp and photographed under UV light using gel documentation system (Alphalmager EC, USA).

Table 2: Nucleotide sequences of selected primers with the number of amplified products and fragment size range (bp)

Primer code	Primer sequence (5′-3′)	No. of amplified products	Fragment size
OPAA-01	CCGGCCCTTC	30	200-1000
OPAA-02	TGCCGAGCTG	35	310-800
OPAA-05	AGGGGTCTTG	32	215-750
OPAA-07	GAAACGGGTG	40	300-830
OPAA-08	GTGACGTAGG	37	210-800
OPAA-11	CAATCGCCGT	55	400-1500
OPAA-15	TTCCGAACCC	42	430-1200
OPAA-17	GACCGCTTGT	34	350-930
OPAA-19	CAAACGTCGG	41	250-735
OPAA-20	GTTGCGATCC	29	435-1025
BG-26	AAGCCTCGTC	30	380-900
BG-27	TGCGTGCTTG	34	290-965
BG-28	GACGGATCAG	41	400-1240
BG-29	CACACTCCAG	38	410-1325
BG-30	TGAGTGGGTG	30	340-950

Cloning and sequencing of the polymorphic band

The putative marker amplified by the random primer OPAA-11 was eluted from 1.2% agarose gel with sterile gel slicer and purified by using Gel Extraction Kit (Nucleopore–Genetix). The sliced amplicon was cloned using the pGEM-T easy vector I (Promega, USA). The ligated plasmid was introduced into *Escherichia coli* strain DH_{10B}, following the protocols for preparing competent cells and transformation using the calcium chloride method [20]. White colonies were picked from LB/ampicillin/X-gal/IPTG plates and grown overnight in LB medium. The plasmid DNA was isolated from the bacterial culture using plasmid isolation kit (RBC Real Genomics™, Taiwan). The inserted fragment was sequenced at Ist Base sequencing, Singapore with SP6 and T7 primer. The nucleotide sequence of 580 bp RAPD amplicon, specific for all the five accessions of *I. ensata* were used for designing primers for development of SCAR marker.

Table 3: Details of the *I. ensata* specific SCAR marker designed from the 580-bp polymorphic sequence

Name of random decamer primer used	Sequence of random decamer primer (5′-3′)	Name of the SCAR primer	Sequence of the SCAR primer (5′-3′)	G+C content (%)	Annealing temperature(°C)
OPAA-11	CAATCGCCGT	Ir-F	AGAGGGGGCCAAGAAGAATA	50.0	58 °C
		Ir-R	TTCAACACGCGAAGTTATCG	45.0	58 °C

SCAR primers designing and validation

Based on the sequence of unique RAPD amplicon a pair of 20 bp oligonucleotide primers (Ir-F and Ir-R), defining a SCAR for each of the cloned fragments was designed and synthesized for specific amplification of the loci identified by the RAPD markers (table 3). PCR reactions were carried out in the 15 µl reaction volume containing (5U µl) Taq polymerase, 1X Taq buffer,(10 mmol)$MgCl^2$, 10 mmol dNTPs (2.5 mmol each), 10 µM of each primer (SCAR-forward and SCAR-reverse) and 50 ng/µl of DNA template. Amplification was performed in a thermal cycler (T100™ Thermal Cycler, Bio-Rad, USA). PCR conditions were set as: initial denaturation 95 °C for 4 min, denaturation at 94 °C for 30 sec, annealing at 58 °C for 45 sec, extension at 72 °C for 50 sec, and a final extension at 72 °C for 2 min and infinite hold on 4 °C. Amplified SCAR product along with 2µl 6X bromophenol dye was loaded on 1-1.2% agarose gel containing ethidium bromide (0.5 µg/ml) prepared in 1X TAE buffer and electrophoresed on 100 volts for one and half hour. The amplified product was visualized on UV lamp and photographed under UV light using gel documentation system Alphalmager EC (USA).

DNA was also isolated from commercial crude drug samples of *I. ensata* using the above isolation method. The isolated DNA samples were then put to test with the above SCAR primers for validation.

RESULTS

DNA extraction and RAPD

High molecular weight genomic DNA was isolated from the dried plant samples. The DNA extraction procedure yielded 600–800 ng of DNA per 100 mg of tissue. First, the isolated genomic DNA showed the absorbance (A260/A280 ratio) of 1.6–1.7 that indicated the contamination of proteins and polysaccharides. This contamination was removed by the Phenol: Chloroform: Iso-amyl Alcohol (25:24:1) treatment which results in the absorbance (A260/A280 ratio) of genomic DNA to the 1.7-1.8 which was highly purified.

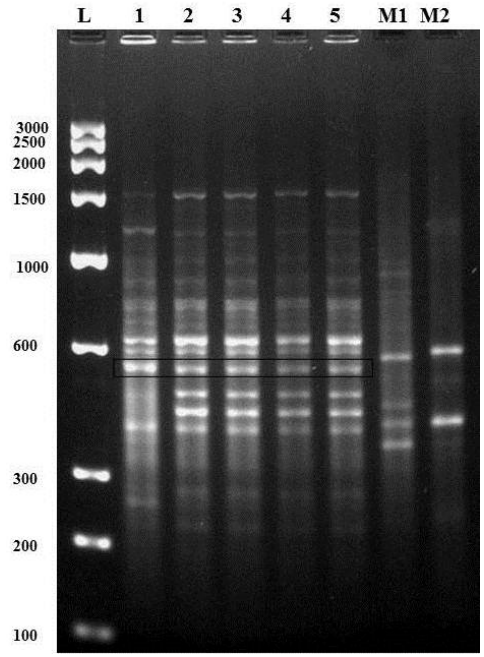

Fig. 1: RAPD pattern with OPAA-11, L-Low range DNA ladder,1-5 DNA Samples of *I. ensata*, M1-M2-Market sample

40 RAPD 10-mer primers were used for screening of all the accessions of the genuine plant (*I. ensata*) and its adulterants. Primers (OPAA 01, OPAA 02, OPAA 05, OPAA 07, OPAA 08, OPAA 11, OPAA 15, OPAA 17, OPAA 19, OPAA 20, BG-26, BG-27, BG-28, BG-29, BG-30) produced good quality, reproducible fingerprint patterns and showed a high level of consistency.

The total of 525 DNA fragments of 200-1500 bp was obtained. To confirm the reproducibility of RAPD pattern the experiment was carried out three times with same conditions, the same composition of reaction volume and a thermal cycler. Primer OPAA-11 consistently amplified an intense 580 bp band that was unique to all the accessions of *I. ensata*. This 580 bp amplicon was not observed in the market samples of *I. ensata* tested. (fig. 1).

Cloning and sequencing of polymorphic band

A polymorphic band (580bp) that was specific in all the accessions of *I. ensata* but absent in its adulterants was selected. This specific band was eluted, cloned and sequenced. Restriction digestion from the *EcoR1* and *Spe1* enzymes revealed 580bp band on 1.2% agarose gel that confirmed the presence of the insert of the desired gene in the vector. The sequencing of this recombinant construct was done by using SP6 and T7 primers.

1
TTTGGCCTTCTCAGATCGTCTATGTGTTCGCGCAGGCCCTGGCCCTCAGG
GGGAACATAT

61
TTCTCACCCTCCTTCGGCAGGATAAAGTTCATCTCCACGAAGTGACGCA
GGTCAAAACCG

121
GACAGGGTATGCTGCATCCGGTAATCCGCCAGGATCATCAGCGGGTCGC
TTTTGGGAACG

181
GCATCCGCGAAGGTTTTCTGATCCGGGAACAGTTTGGCGCTCTGAACGT
CATTAAAGAGG

241
GGGCCAAGAAGAATATCAGGTGAGCTTTGCTGGCTCGTTGGCTGGTCT
TCAGCATAACCA

301
AATGCGGTAACACCGAGTAGTGCGCCTGCCAACGCAAGGGGTAAAAAA
GCGGGGCGTCGT

361
GCGCGAGGTCTTATCATCGGTTCGTCTCCTGTCTTCACTGAGCAGCGTG
ACCGCTGTTGT

421
CAATTACCTGAAAACCTTAGACAAATATCGATAACTTCGCGTGTTGAA
TGTCCCATTTTT

481
CGCATAACCAGATAATTCGGGTGCGCTTTCACTACACCAGGGTTGGATT
ACTTAATTTTT

541
AACCACACCTCGGAAAGGCCGGGTAAGCTTCATGCCACCCGGCTTAAGC
CCTACAACCCT

601
TTATTATCCATCAGCACCGCCAAATCCACCAGACGATCTGAGAAGGCCA
AAA

Validation of SCAR primers

The genomic DNA of all the accessions of *I. ensata* and of the market samples were amplified by the SCAR primers (Ir-F and Ir-R) for the

applicability of the SCAR marker for the molecular authentication of the plant. A single, bright and distinct band of 260 bp was obtained only from the genomic DNA of the authenticated accessions of the *I. ensata* and no amplification product was obtained from the genomic DNA of the market samples of *I. ensata* (fig. 2). This confirms the specificity and the sensitivity of the SCAR marker for the *I. ensata*. Further, this result confirms that these SCAR markers can be used for the molecular identification of *I. ensata*.

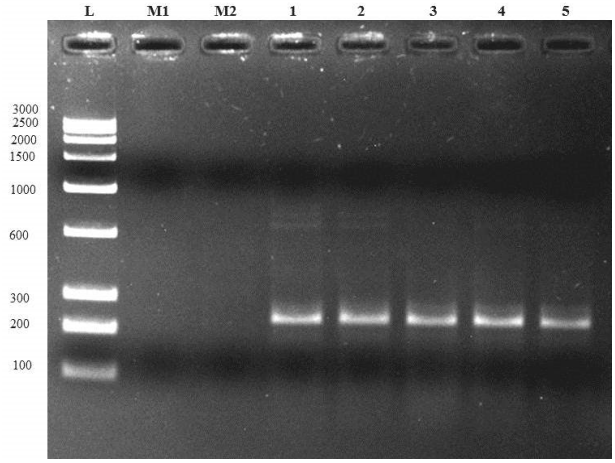

Fig. 2: PCR amplification of *I. ensata* and market sample with SCAR (Ir-F andIr-R) primers, L-DNA Marker, M1-M2-Market sample,1-5 DNA Samples of *I. Ensata*

DISCUSSION

In this study, we developed the RAPD based SCAR marker for the identification of the *I. ensata*. For the development of SCAR marker, we used five accessions of the plant as it is the high altitude plant and has very low availability. However, many published studies showed the development of RAPD based marker from the large number of accessions but these plants have good availability such as genus *Artemisia* [21], *Panax* species [22], *Phyllanthus* species [23], *Ganoderma lucidum* [24], *Ipomoea mauritiana* [25], *Bemisiatabaci* (Genn.) [26]. additionally, in our study, we used dried root samples for the isolation of the genomic DNA and we successfully isolated the ample amount of genomic DNA by doing some modifications in the CTAB method of Doyle and Doyle (1990). Thus, establishing a reliable DNA fingerprinting for the dried samples of the *I. ensata* is very significant for SCAR analysis. In our RAPD analysis, significant genetic polymorphism was observed among *I. ensata* and its adulterants. The SCAR primers designed using this sequence variation was found to be specific for *I. ensata* making the technique more stringent and specific as compared with RAPD marker.

However, at the molecular level, there are few studies that have been reported for the different species of *Iris* [27-28]. By using RAPD, genetic diversity has been analyzed amongst different species of *Iris* [27]. Based upon a study on *Iris* species, the phylogeny of the genus *Iris* has been reported to be based on matK gene and trnK intron sequence data [29]. Yue-E-Xiao *et al.* (2012) developed 13 polymorphic microsatellite loci in *I. ensata* exhibited a large number of alleles per locus and high heterozygosity [29]. Although, some work has been reported on the genus *Iris* at the molecular level, but very few on *Iris ensata*. As we discussed earlier our study provide an easy and cheaper way of identification of *I. ensata* from their adulterants, as RAPD based SCAR marker is easy to develop and it is economically sound too.

CONCLUSION

In the present study, RAPD based sequence characterized amplified regions (SCAR) marker of *Iris ensata* Thunb. The plant was successfully developed, which is more accurate than RAPD markers. Therefore, the SCAR markers (Ir-F and Ir-R) developed in the present study could be of immense use for the identification of *Iris ensata* Thunb. a medicinally important plant.

AUTHOR'S CONTRIBUTION

MM performed all the experiments of the manuscript and wrote the manuscript. SRJ assisted MM in the experiments as well as in the writing the manuscript. AA and JA were instrumental in helping the students in carrying out this work and final proofreading of this manuscript was also done by them. The working concept was designed by AA.

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CONFLICT OF INTERESTS

Declared none

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