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Development of ISSR-Derived SCAR marker for rapid and accurate authentication of West Indian arrowroot (*Maranta arundinacea* L.)

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Abstract

Arrowroot, or West Indian arrowroot (*Maranta arundinacea* L.), native to the Antilles, Mexico, and other Central American countries, has the potential to become a flour source. Species are primarily identified based on morphological characteristics, which are influenced by the environmental factors. Currently, the most accurate authentication method is through DNA markers or molecular fingerprinting. Arrowroot accessions from various provinces were distinguished from their related species using Inter-Simple Sequence Repeat (ISSR)-derived Sequence-Characterized Amplified Region (SCAR) markers. DNA profiling was conducted based on the results of DNA amplification using ISSR primers. The polymorphism data identified a fragment, UBC825Ma-924, which was generated by primer UBC825 and was only present in arrowroot genotypes. The specific band was sequenced, and SCAR primers (SC-UBC825Ma-630) were designed and synthesized to amplify the 630 bp band. Using the primer pair and total DNAs of *M. arundinacea* and related species, diagnostic PCRs produced specific amplification only in *M. arundinacea* genotypes. This approach is quick, reliable, simple, viable for authentication, and helpful for cultivar development and conservation.

Keywords: Inter-Simple Sequence Repeat (ISSR) Marker, Local Tuber Crop, *Maranta arundinacea* L., Multiplex Polymerase Chain Reaction (PCR), Sequence-Characterized Amplified Region (SCAR) Marker, Species-Specific Marker

1. Introduction

Maranta arundinacea L., a member of the genus *Maranta* and the Marantaceae family, goes by several names depending on its location. These include "Arrowroot," "Arrowhead," "Bermuda Arrowroot," "Maranta," "St. Vincent Arrowroot," "West Indian Arrowroot," and "Araruta" in Portuguese [1]. This erect, perennial, large herb has white flowers that very rarely produce seeds. The plant has a perennial, fibrous, starchy rhizome with numerous tufted, fleshy, scaly tubers emerging from the crown [2]. Arrowroot flour is easily digested, has a low glycemic index, and high fiber content, making it a food with high economic and nutritional value [3], particularly for young children, the elderly, and those with digestive diseases [4]. The rhizomes can be boiled or roasted for consumption [5].

Arrowroot flour is used as a thickening agent in the pharmaceutical, food, and cosmetics industries [6]. Arrowroot rhizomes are difficult to distinguish when dormant, and their morphological characteristics differ greatly depending on the growing environment [7]. Identifying arrowroot species is crucial for growers, farmers, distributors, merchants, the food sector, and consumers [8]. Therefore, it is important to create an approach that can quickly, accurately, and unambiguously distinguish arrowroot and related species. DNA fingerprinting, invented in 1985 [9], is utilized in criminal investigations. Several DNA-based molecular marker techniques have been developed, including the analysis of restriction fragment length polymorphism (RFLP) using restriction enzymes, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), and single nucleotide polymorphism (SNP) detection via polymerase chain reaction (PCR) [10-12]. However, the reproducibility of RAPD and AFLP techniques presents limitations, and the markers they generate are dominant, leading to constraints in assessing relationships among genetic

resources [13]. Inter-Simple Sequence Repeat (ISSR) is a molecular marker commonly utilized in various applications such as genetic fingerprinting [14], species or variety identification [15], phylogenetic analysis [16], and hybridization analysis [17]. A single 16–25 bp primer targeted at identical regions among microsatellites is employed as the ISSR marker. The primers consist of a nucleotide anchoring means that target the microsatellite regions comprised of up to eight repeating dinucleotide units, six repeating trinucleotide units, or multiple repeating tetra- or penta-nucleotide units [18].

Only a few reports have been published on ISSR analyses of arrowroot germplasm, including species identification and genetic diversity [19-22]. Our study used a PCR method with ISSR markers to develop more accurate and stable SCAR primers to distinguish arrowroot genotypes and related species from four provinces in Thailand. Based on the ISSR analysis, novel SCAR markers and combination primers were investigated to accurately and effectively distinguish related species with arrowroot genotypes by PCR.

2. Materials and methods

2.1 Plant materials

The samples were collected between May and August 2022 from naturally growing flora in four provinces in Northeast Thailand. Three *M. arundinacea* accessions, three *M. leuconeura* "Kerchoveaus" accessions as related species, and one *Canna edulis* Ker. as an outgroup genotype were collected from different locations, including Mahasarakham, Nakhon Ratchasima, Phitsanulok, and Loei provinces (Table 1, Figure 1(A)-1(D)). The whole rhizome of the arrowroot was collected from each accession and then replanted in the greenhouse of the Department of Biology, Faculty of Science, Mahasarakham University. Species identification was performed using the identification key of Woodson and Schery, Wu and Kennedy, and Lim [23-25]. Species identification was also conducted through interviews with arrowroot farmers. Fresh young leaves about 5 months old were taken from each accession for genomic DNA extraction.

Table 1 The plant accessions subjected for this study.

No.	ID code	Name	Collection location
1	Acc. 1	*M. arundinacea*	Mahasarakham
2	Acc. 2	*M. arundinacea*	Nakhon Ratchasima
3	Acc. 3	*M. arundinacea*	Phitsanulok
4	Acc. 4	*M. leuconeura*	Mahasarakham
5	Acc. 5	*M. leuconeura*	Loei
6	Acc. 6	*M. leuconeura*	Phitsanulok
7	Acc. 7	*C. edulis*	Mahasarakham

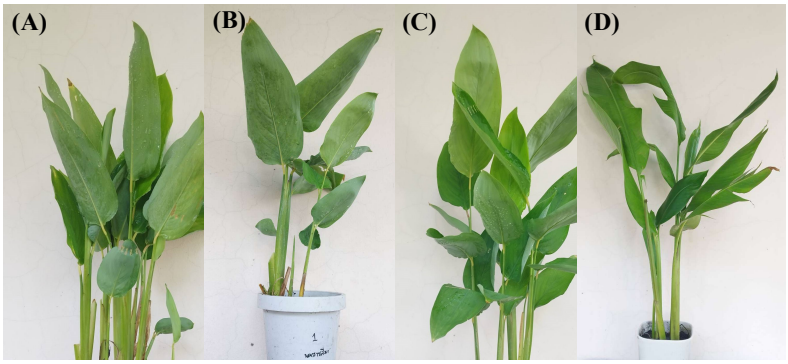

Figure 1 The three arrowroot accessions and one related species investigated in this study are: (A) *M. arundinacea* (Mahasarakham, Acc. 1), (B) *M. arundinacea* (Nakhon Ratchasima, Acc. 2), (C) *M. arundinacea* (Phitsanulok, Acc. 3), and (D) *C. edulis* (Mahasarakham, Acc. 7).

2.2 Genomic DNA Isolation

The molecular analysis was performed in the Laboratory of Molecular Genetics, Department of Biology, Faculty of Science, Mahasarakham University, Thailand. The cetyltrimethylammonium bromide (CTAB) method was partially adapted and utilized for small amounts of tissue to extract genomic DNA from young leaf tissue [26,27]. Samples (0.2 g) of fresh young leaves were ground in liquid nitrogen, and the homogenate was then added to 1 ml of extraction buffer [2% CTAB (hexadecyltrimethylammonium bromide), 100 mM Tris-HCl

buffer, 20 mM EDTA, 1.4 M NaCl, 1% PVP-40, pH 8.0] containing 1 µl of 2-mercaptoethanol, which was added just before use. The extract was incubated at 60°C for one hour with occasional swirling, combined with an equal amount of chloroform: isoamyl alcohol (24:1, v/v), and centrifuged at 13,000 g for 10 min. The aqueous phase was moved to a fresh tube and combined with 2/3 volume of ice-cold isopropanol. The mixture was left at -20°C for 20 min and then centrifuged again at 13,000 g for 10 min. The pellet was rinsed with 10 mM ammonium acetate in 76% ethanol, allowed to dry for 1 to 2 hours at room temperature, and dissolved in 40 µl TE buffer (10 mM Tris–HCl pH 8.0, 0.1 mM EDTA). The DNA solution was treated with 1 µl of Ribonuclease A (RNAse A) (10 mg/ml) and heated to 37°C for 10 min to remove Ribonucleic acid (RNA). The extracted DNA was analyzed using 1% agarose gel electrophoresis, and its quantity was estimated using a spectrophotometer to measure the absorbance at 260 and 280 nm. According to the readings of the spectrophotometer, 50 ng/µl DNA solutions were prepared and utilized for PCR amplification.

2.3 ISSR analysis

Five ISSR primers were used to carry out ISSR analysis according to the protocol described by Borse et al. [28]. The ISSR primers (University of British Columbia (UBC), Biotechnology Laboratory, Vancouver, BC, Canada) were chosen from previous studies and included UBC-811, UBC-817, UBC-818, UBC-825, and UBC-827 (see Table 2). The ISSR-PCR was conducted using a 25 µl reaction volume containing 0.2 mM dNTP mix (Vivantis Technologies, Malaysia), 2 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Invitrogen, Brazil), 1X *Taq* polymerase buffer (Invitrogen, Brazil), 1.5 µM of each ISSR primer (Integrated DNA Technologies, Singapore), and 50 ng of genomic DNA. DNA amplifications were carried out using a programmed thermal cycler (Applied Biosystems VeritiTM, Singapore) with an initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, annealing at 45–53°C for 45 sec, and extension at 72°C for 1 min. A final incubation at 72°C for 4 min was used to complete primer extension. Each primer had a specified annealing temperature (Table 2). Gel electrophoresis in 1% agarose gel with 1X TBE buffer was used to separate the amplification results. DNA was stained with 0.5 µg/ml non-toxic DNA dye (Red Safe™, iNtRON Biotechnology, Inc., South Korea) and imaged through a UV-Vis transilluminator.

The amplification products were electrophoretically separated on 1.5% agarose gels with 1X TBE (40 mM Tris-borate, 1 mM EDTA) containing 0.5 µg/ml non-toxic DNA dye, and then visualized on a UV transilluminator. A comparison with a 100 bp DNA ladder size standard (Vivantis Technologies, Malaysia) was used to estimate the size of the amplified products. Among these, the primer UBC825 (5'-ACACACACACACACACT-3') consistently produced a single, intense band of 924 bp in samples of the species *M. arundinacea*, while this band was not present in other genotypes (Table 2). The band, designated UBC825Ma-924, was selected as a putative species-specific marker of *M. arundinacea*. These ISSR fragments, designated UBC825Ma-924, were excised from agarose gels and extracted using a NucleoSpinTM Gel and PCR Clean-up Kit (Macherey-Nagel GmbH & Co., Germany), following the manufacturer's recommendations. The recovered DNA fragments were sequenced using an Applied Biosystems 3730XL DNA Analyzer and the ABI PRISM dye terminator cycle sequencing kit at Ward Medic Ltd., Bangkok, Thailand.

Table 2 Primer sequences, annealing temperatures and product length in base pairs of the ISSR markers utilized in this study.

Markers	Sequence (5'- 3')	Annealing temperature (°C)	Size range (bp)	References
UBC 811	GAG AGA GAG AGA GAG AC	53	300-2,000	[16]
UBC 817	CAC ACA CAC ACA CAC AA	47	200-900	[16]
UBC 818	CAC ACA CAC ACA CAC AG	45	350-1,600	[16]
UBC 825	ACA CAC ACA CAC ACA CT	52	400-2,000	[16, 19]
UBC 827	ACA CAC ACA CAC ACA CG	52	300-1,600	[16]

2.4 Design and synthesis of SCAR primers

According to the UBC825Ma-924 ISSR amplicon's sequencing data, the specific SCAR primers were developed using the primer design tool available online [29]. A primer pair was custom synthesized to design the SCAR markers. SC-UBC825Ma-630_F was designed as a forward primer and SC-UBC825Ma-630_R as a reverse primer. Synthesis of these primer pairs was performed by Integrated DNA Technologies PTE, Singapore.

2.5 SCAR marker analysis and multiplex PCR

SCAR primers were developed for PCR based on ISSR analysis to identify the genotype of *M. arundinacea*. The SCAR primers, SC-UBC825Ma-630_F (5'-GTGTCTGGAGCCGAGTTCAA-3') and SC-UBC825Ma-630_R (5'-AGACGCGTTGACTGTGCTTA-3'), were used to amplify DNA derived from seven specimens, including

three genotypes of *M. arundinacea* and related species such as *M. leuconeura* from three provinces of Thailand, and *Canna edulis* Ker. as an outgroup genotype. A 25 µl aliquot of the final reaction volume contained 0.2 mM dNTP mix (Vivantis Technologies, Malaysia), 2.0 mM $MgCl_2$ (Vivantis Technologies, Malaysia), 0.08 U *Taq* DNA polymerase (Invitrogen, Brazil), 1X *Taq* polymerase buffer (Invitrogen, Brazil), 0.5 µM SCAR primer (Integrated DNA Technologies, Singapore), and 10 ng of genomic DNA. Each run included negative controls to check for contamination, using distilled water in place of the DNA. Amplification of PCR was carried out on a programmed thermal cycler (Applied Biosystems VeritiTM, Singapore). The template DNA was initially denatured for 2 min at 94°C and then underwent 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C, with a final extension for 4 min at 72°C, followed by holding at 4°C until recovery.

The amplification products were electrophoresed on 1.5% agarose gels containing 0.5 µg/ml non-toxic DNA dye, and UV transilluminator images were taken. Multiplexed primers using these SCAR primers for PCR and 18S rDNA primers (18S-F, 5′-GACTGTGAAACTGCGAA-3′ and 18S-R, 5′-ATACGCTATTGGAGCTGGA-3′) [30-32] were applied in 25 µl reaction mixtures with the same components as in the single analysis, except the 18S rDNA primers, which were added at a concentration of 1.0 µM. Thermocycling conditions for amplification with multiplexed primers were carried out following the amplification of SCAR primers as mentioned above. To verify the SCAR markers, the DNAs were amplified twice.

3. Results

3.1 ISSR-PCR and identification of specific ISSR amplicons

Five ISSR primer pairs were used to evaluate seven specimens of *M. arundinacea*, *M. leuconeura*, and *C. edulis* to identify polymorphic bands among the sample genotypes (Table 2). These ISSR produced 48 amplicons and showed polymorphic bands across the genotypes of the sample. Only the primer UBC825 (5'-ACACACACACACACACT-3'), yielded a DNA amplicon that was consistently produced as a single, strong band with a size of 924 bp only in the *M. arundinacea* genotype and was not present in the remaining genotypes. The identified band, designated UBC825Ma-924, was shown to be a potential *M. arundinacea*-specific ISSR marker (Figure 2).

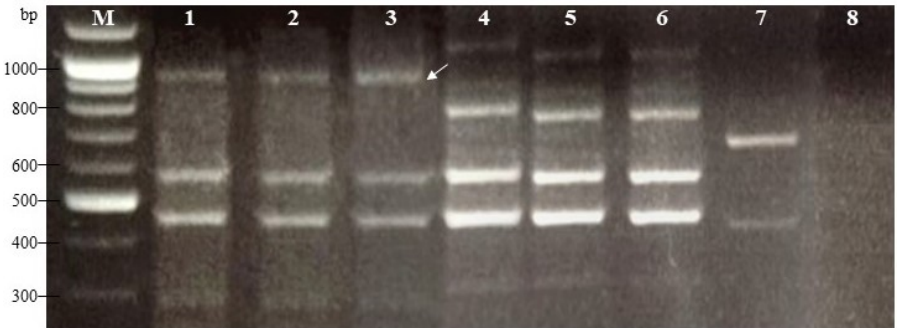

Figure 2 A sample figure of DNA polymorphism between *M. arundinacea* and related species using the ISSR primer (UBC825): lane 1, *M. arundinacea* (Acc. 1); lane 2, *M. arundinacea* (Acc. 2); lane 3, *M. arundinacea* (Acc. 3); lane 4, *M. leuconeura* (Acc. 4); lane 5, *M. leuconeura* (Acc. 5); lane 6, *M. leuconeura* (Acc. 6); lane 7, *C. edulis* (Acc. 7) lane C, Negative control and lane M, DNA molecular weight marker, respectively. The arrow indicates a specific DNA fragment of *M. arundinacea* genotypes.

3.2 Sequencing ISSR fragments

The ISSR fragment of UBC825Ma-924 was removed from agarose gels and extracted as previously mentioned. The obtained DNA fragments were then sequenced on an Applied Biosystems 3730XL DNA Analyzer. According to the sequencing results, the ISSR fragment UBC825Ma-924 had an exact size of 924 bp. A 20-mer oligonucleotide primer pair was synthesized for use in PCR reactions based on the sequence information obtained from the identified ISSR band (Table 3).

Table 3 Sequences of the ISSR fragment UBC825Ma-924 utilized to develop the SCAR marker SC-UBC825Ma-630 specific to the genotype of *M. arundinacea.*

bp	Sequences of ISSR fragment	bp
1	AGCTGTGTGTGTGTGTGACCAGGTCTAAATTGATTGGGCTGAAGCAGAGACGCCGGCGCAC	63
64	CTTGACTCGGGATGCCCTAGTTTCCTGCTGCGTTCTGACTCTGGTATAACTCGGTGGAAAACC	126
127	TTCTAGAGTTCAGTAGACAGAATTTAGATCGATGAA**GTGTCTGGAGCCGAGTTCAA**GGGAGCA	189
190	ACTTCACACATCATTGTATGATGAACAACGAATCTGAAGAATAAGGAAATCCGTGCGCTCCAC	252
253	CAACATCAGCCAAGATATAGACATTTCCGCAACCGAAGAAACGGAGATGCCGGTTTACATTTT	315
316	TTCGAAACTAACAACCCATTTTTCATTTTGGAATCTGATTTTGCTAGATTTGTATTGGAAAAT	378
378	TGCTGATGCATTAGGGGAATTCCCCGCATCCAACTGATCATAGTCCTTCTCCGTGGAGACTTT	441
442	GCCCATTCCATGTTGAAGGCCTCACCTCTGTATAGAAACGACTTTGTTTAACTCCAAATGCTA	504
505	CCCAGGTCAGCTTGCACAAAAGTTCTCCAGAGCTTTGTGGGAATAAGAGTTGAACGGTGCTTG	567
568	TTTTCTAGAGGCACAGGACAGAGGAAAGCGTTCCTGCTGCACGGAGGGCGATGCGGTCCAATA	630
631	CTGAACGTGAGAAGGCGCAGTGCCATAAAATATGAGTAGAGGTCGCCTCCTCGTGTCGGCACC	693
694	CATGACAGCATACATCATGTTGCTTTCCTTTTCGCACCATCATGGCTTTTGTGTTGAGTCTAT	756
757	CAATCAAGATAAGCCA**CAGTGAAGTTGATGCTTGAG**ATGATAAAGCTGCACCAGACGTCTTTA	819
820	CCATAAGCACAGTCAACGCGTCTATGACCGAGAAACTTATATTGCGAGGCTACACTGAAGCGA	882
883	CATTTACTACTCCATCTCACACACACACACACAGGATATCAG	924

Note: ISSR primer (UBC825) sequences are underlined with positions of SCAR primers in bold.

3.3 Design and synthesis of SCAR primers

The developed SCAR primer pair of SC-UBC825Ma-630_F (5'-GTGTCTGGAGCCGAGTTCAA-3') and SC-UBC825Ma-630_R (5'-CAGTGAAGTTGATGCTTGAG-3') was used for amplification of genomic DNA from the seven specimens of *M. arundinacea*, *M. leuconeura*, and *C. edulis*. Thermocycling conditions for SCAR primer amplification were optimized as previously noted. Only the DNA of *M. arundinacea* specimens yielded a single, obvious, and clearly defined band of 630 bp, while neither the outgroup genotype nor any other closely related species' DNA showed nonspecific amplification (Figure 3).

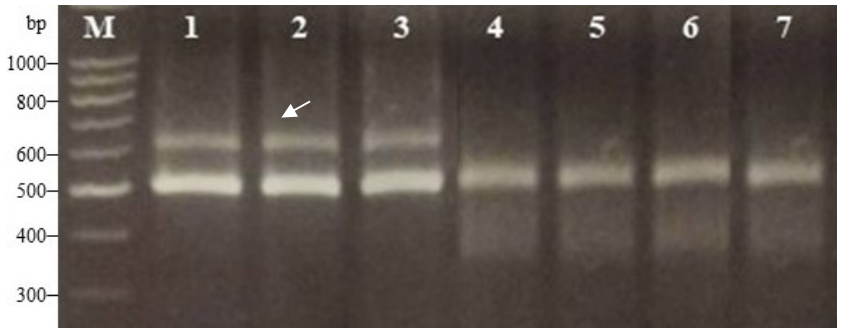

Figure 3 PCR results utilizing the SCAR primers, SC-UBC825Ma-630 and 18S rDNA primers: lane 1, *M. arundinacea* (Acc. 1); lane 2, *M. arundinacea* (Acc. 2); lane 3, *M. arundinacea* (Acc. 3); lane 4, *M. leuconeura* (Acc. 4); lane 5, *M. leuconeura* (Acc. 5); lane 6, *M. leuconeura* (Acc. 6); lane 7, *C. edulis* (Acc. 7) and lane M, DNA molecular weight marker. The arrow indicates a specific DNA fragment of *M. arundinacea* genotypes.

3.4 SCAR marker analysis and multiplex PCR

To evaluate the feasibility of using such a method for identifying *M. arundinacea*, a polymorphic band obtained with the UBC825(5'-ACACACACACACACACT-3') was selected and sequenced to create SCAR markers. The initial ISSR marker was subsequently converted into more an accurate SCAR assessment (SC-UBC825Ma-630_F and SC-UBC825Ma-630_R). The SCAR markers identified variations between the seven genotypes of *M. arundinacea*, *M. leuconeura*, and *C. edulis*. SC-UBC825Ma-630_F and SC-UBC825Ma- 630_R produced a DNA fragment of 630 bp only in *M. arundinacea* genotypes. The practical use of markers was demonstrated by the observation that not one of the other *M. arundinacea* samples amplified non-specific DNA fragments from these SCAR loci. Figure 2 shows the results of DNA amplification employing the SCARs on seven samples of *M. arundinacea*, *M. leuconeura*, and *C. edulis*. These demonstrated polymorphisms that could be utilized for distinguishing species and were related to the original ISSR polymorphism. The SCAR primer and 18S rDNA primer combinations were adopted for analyzing the amplifications of seven specimens of *M. arundinacea*, *M. leuconeura*, and *C. edulis*. A combination of primers yielded DNA fragments of 630 bp being

produced by the SCAR markers only in *M. arundinacea* genotypes since other specimens could not be amplified and detected with agarose gel electrophoresis. Moreover, confirmation of the validity of the multiplexed primers was established by PCR observed in the DNA bands of around 500 bp amplified by 18S rDNA primers, which were present across the seven genotypes (Figure 3).

4. Discussion

The synthesis of SCAR primers, subsequently used to identify genotype taxonomy, can be based on the sequencing of genotype-specific ISSR markers. In this investigation, three regional arrowroot (*M. arundinacea*) cultivars from three provinces in Thailand focused to identify genotype-specific marker. The related genotypes, *M. leuconeura* and *C. edulis*, were used as outgroups to provide a comparative reference for assessing the genetic relationships among the arrowroot accessions. Comparing genetic markers between arrowroot germplasms and related species helps identify unique genotype-specific markers, thereby enhancing the accuracy and reliability of genetic analysis [33]. Molecular analysis using ISSR markers successfully revealed polymorphism and genetic variability between the local arrowroot species and other related genotypes. The identified suitable ISSR amplicons were efficiently and reliably transformed into more specific SCAR markers. Several researchers have explored applications to identify arrowroot species and their genetic variability. For example, the genetic diversity of arrowroot (*M. arundinacea*) germplasm was assessed using ISSR markers [17,20], while Kumar et al. and Lui et al. converted ISSR markers to SCAR markers, that provided applicable sequence-specific markers for germplasm identification and discrimination [30,31].

ISSR-PCR is widely utilized to analyze plant genomes, but its practical applicability can be enhanced by sequencing the ISSR products and converting them into more specialized assays. Also, employing the direct sequencing analysis makes such conversion more economically feasible [34]. To assess the efficacy of such a method for identifying local arrowroot, a polymorphic band of 924 bp was discovered using the ISSR primer UBC825 that was only present in *M. arundinacea* genotypes but absent in other samples. This band with the designation UBC825Ma-924 was chosen for sequencing and for synthesizing specific SCAR markers. In comparison to the ISSR marker, SCAR was more precise and reliable because it used two sets of 20 bp oligonucleotide-specific primers, SC-UBC825Ma-630_F and SC-UBC825Ma-630_R, to amplify the 630 bp fragments of the targeted regions from genomic DNA under stringent circumstances. The capability to create internal primers enhanced the usefulness of the developed markers and enabled accurate multiplexing. SCAR, SC-UBC825Ma-630 which generated exactly one band (Figure 3), rendering it suitable for plus-minus screening in the electrophoregram following PCR. We found that only the genotypes of *M. arundinacea* were fragmented and no amplification was observed in any of the other samples. Therefore, this SCAR can be utilized for arrowroot seedling identification and marker-assisted selection; however, it has to be verified by screening a wider variety of species and cultivars. The recommended approach thus provides a straightforward and dependable strategy for developing efficient and accurate SCAR markers that could help differentiate regional Thai arrowroot germplasm. The discovery of species-specific markers has become an important goal for paternity testing, genotype identification, cultivar improvement, as well as genetic conservation of regional tuber plants.

5. Conclusion

Arrowroot cultivars, *M. arundinacea*, along with related genotypes were utilized to synthesize SCAR primers based on genotype-specific ISSR markers. DNAs were amplified using ISSR primers to generate profiles, which were analyzed for polymorphism. UBC825Ma-924 fragment, generated by primer UBC825, was specific to arrowroot genotypes. The fragment was sequenced, and SCAR primers (SC-UBC825Ma-630) were developed, and synthesized to amplify the 630 bp band. Diagnostic PCRs using the SCAR amplified only *M. arundinacea* genotypes. This approach provided a rapid, reliable, and cost-effective method for identifying the genotypes of *M. arundinacea*. Therefore, molecular tools assisted in preserving plant diversity while supporting sustainable agriculture.

6. Acknowledgements

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