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Development of ISSR derived SCAR marker for economically important *Benstonea thwaitesii* (Martelli) Callm. and Buerki

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Benstonea thawaitesii (Martelli) Callm. and Buerki, an economically important plant of Pandanaceae family, is having fragrant male inflorescence. In vegetative growth phase, it is difficult to distinguish *B. thwaitesii* from other Pandanaceae species due to similar morphological characters. In the present study, we have developed inter simple sequence repeat (ISSR) derived sequence characterized amplified region (SCAR) marker for identification of *B. thwaitesii*. A set of 15 Indian Pandanaceae species was screened using 15 ISSR primers. Among 15 primers, UBC-857 generated unique 1 kb band specific to *B. thwaitesii*. It was further cloned, sequenced and used to design SCAR primer that amplified the expected amplicon of 750 bp in *B. thwaitesii* only. This is the first report of developing species-specific SCAR marker in *B. thwaitesii* using ISSR primers.

Key words: *Benstonea thwaitesii*, Pandanaceae, ISSR, SCAR

Introduction

The Pandanaceae is a monocotyledonous, paleotropical family comprise nearly about 700 species and 5 genera. Amongst them, genus *Pandanus* has the broadest geographical distribution, occurring throughout the Old World tropics followed by genus *Benstonea* distributed widely from India to the South Pacific, with high species richness in South East Asia[1]. Southern and North-Eastern India represents 15 Pandanaceae species, in which two economically important species *Pandanus odorifer* (Forssk) Kuntz and *Benstonea thawaitesii* (Martelli) Callm and Buerki are exploited for essential oils[2-4]. It grows extensively along the fresh water streams in forests of Maharashtra, Goa and Karnataka[5]. Staminate inflorescences of *B. thwaitesii* have high level of fragrance[6]. Most of Pandanaceae species are morphologically similar and can be confirmed taxonomically only after attaining maturity. It is very difficult to distinguish *B. thwaitesii* from other Pandanaceae species in vegetative stage. Sequence characterized amplified region (SCAR) marker is fragment of genomic DNA at a single genetically defined locus identified by PCR amplification using specific oligonucleiotide primers[7] and used as a tool in identifying the species. In the present investigation, inter simple sequence repeat (ISSR) markers were used for development of SCAR marker in *B. thwaitesii* to facilitate its efficient and accurate identification.

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Materials and Methods

Plant Materials and DNA Extraction

Fifteen Indian Pandanaceae species, 13 from genus *Pandanus* (*P. odorifer* (Forssk) Kuntz, *P. kaida* Kurz, *P. unipapillatus* Dennst., *P. amaryllifolius* Roxb., *P. furcatus* Roxb., *P. palakkadensis* Nadaf, Zanan & Wakte, *P. mangalorensis* Nadaf & Zanan, *P. dubius* Spreng., *P. emerginatus* St. John., *P. diversus* St. John., *P. nepalensis* St. John., *P. martinianus* Nadaf & Zanan and *P. unguifer* Hook. f.) and 2 from genus *Benstonea* (*B. thwaitesii* (Martelli) Callm. & Buerki and *B. foetida* (Roxb.) Callm. & Buerki) were collected from their native habitats of Western Ghats and North Eastern Himalaya. The seedlings were planted in Botanical Garden, Department of Botany, University of Pune, Pune. Genomic DNA was isolated from fresh leaves using CTAB method with minor modifications[8]. The quality and quantity of the isolated DNAs were determined on 1% agarose gel and λ-DNA (Fermentas, USA) was used as a standard. Ethidium bromide used as visualizing agent and the gel was documented using gel documentation system (Bio-Rad, California, USA).

ISSR-PCR Amplification

Fifteen isolated DNA of Indian Pandanaceae species were assessed using inter simple sequence repeat (ISSR) markers. Primers from UBC-SSR set 9 (Biotechnology Laboratory, the University of British Columbia, Canada) were used for ISSR analysis. A total of 15 ISSR primers were screened for SCAR marker development (Table 1). ISSR-PCR was performed in total 25 µl reaction volume contained 0.2 mM of each dNTP (Fermentas, USA), 2 mM $MgCl_2$ (Invitrogen, Brazil), 1U *Taq* DNA polymerase (Invitrogen, Brazil), 1X *Taq* polymerase buffer (Invitrogen, Brazil), 1.5 µM primer and 50 ng of genomic DNA. The DNA amplifications were carried out in thermal cycler (Corbett Research, Australia) with an initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, annealing at 48°C for 45 sec, extension at 72°C for 1 min and a final extension at 72°C for 5 min. PCR products were resolved on 1.5% agarose gel in 1X TAE buffer. The DNA was stained with ethidium bromide (10 µg/ml) and visualized under gel documentation system (Bio-Rad, USA).

Cloning and Sequencing of Specific ISSR Amplicon

Distinctly amplified fragment was excised from the agarose gel and purified using gel extraction kit (Promega, USA) as per manufacturer's recommendations. The eluted DNA fragment checked on 1.2% agarose gel and ligated into pGEM® -T Easy Vector (Promega, USA) following the manufacturer's instructions. Ligated plasmid was transformed in *Escherichia coli* strain JM109 following the calcium chloride method[9]. The recombinant plasmid DNA was isolated from the bacterial culture using alkaline lysis method and purified[10-11]. The recombinant plasmid was sequenced commercially using vector specific primers (T7 forward and SP6 reverse).

Designing of SCAR Primer

Sequence of amplified PCR product of *B. thwaitesii* was used for primer designing using online primer designing tool (http://www.ncbi.nlm.nih.gov/tools/ primer-blast/). Based on sequence of ISSR (UBC-857) amplicon, two primer pairs were custom synthesized for development of SCAR marker. FT1 and FT2 were designed as the forward and RT1 and RT2 were designed as reverse primers (Table 2).

Conversion of Polymorphic ISSR Marker into SCAR

Genomic DNA of *B. thwaitesii* was amplified using above mentioned primers. The amplification reaction contained 0.2 mM of each dNTP (Fermentas, USA), 2 mM $MgCl_2$ (Invitrogen, Brazil), 1U *Taq* DNA polymerase (Invitrogen, Brazil), 1X *Taq* polymerase buffer (Invitrogen, Brazil), 10 pM primers of each forward and reverse, 50 ng of genomic DNA and final volume was made to 25 µl with distilled water. Amplification was performed in a DNA thermal cycler (Corbett Research, Australia). The reaction conditions for PCR consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 sec, annealing at 50 and 57°C for 30 sec respectively, extension at 72°C for 1 min and a final extension at 72°C for 5 min. PCR products were resolved on 1.5% agarose gel in 1X TAE buffer. The DNA was stained and visualized as mentioned above.

Validation of SCAR Marker

The validation of resulting FT1 and RT1 SCAR markers was performed over Indian Pandanaceae species mentioned elsewhere following the same amplification reaction contents and PCR conditions.

Results

Amplification, Cloning and Sequencing of *B. thwaitesii* Specific ISSR Amplicon

Among the 15 screened ISSR primers for SCAR marker development, UBC-857 consistently amplified a polymorphic fragment of 979 bp in *B. thwaitesii*, which was absent in the other species (Fig. 1). The sequence of amplified fragment is depicted in Figure 2.

Amplification and Validation of SCAR Marker

FT1 and RT1 primer pair amplified expected amplicon (760 bp) where as FT2 and RT2 did not

Table 1 — List of ISSR primer used for SCAR analysis

Primer	Sequences (5'-3')	Primer	Sequences (5'-3')
UBC-830	$(TG)_8G$	UBC-857	$(AC)_8YG$
UBC -835	$(AG)_8YC$	UBC-860	$(TG)_8RA$
UBC-840	$(GA)_8YT$	UBC-866	$(CTC)_6$
UBC-841	$(GA)_8YC$	UBC-881	$(GGGTG)_3$
UBC-842	$(GA)_8YG$	UBC-888	$BDB(CA)_8$
UBC-845	$(CT)_8RG$	UBC-843	$(CT)_8RA$
UBC-853	$(TC)_8RT$	UBC-847	$(CA)_8RC$
UBC-855	$(AC)_8YT$		

B = C, G or T; R = A or G; Y = C or T; D = A, G or T

Table 2 — Sequence of SCAR primers

ISSR primer	SCAR primer	No. of base pairs	Sequence (5'-3')
UBC-857	FT1	15	CACACACACCGACGC
	RT1	15	CTTGGAATTGGGCGG
	FT2	20	GCCGTTGAGGAAAAGAACAG
	RT2	20	TGGAGAGGAGATTTCGATGG

amplified expected amplicon. The FT1 and RT1 primer was validated by applying it on the DNAs of Indian Pandanaceae species that showed single and sharp 760 bp band in *B. thwaitesii* whereas remaining Pandanaceae species didn't show any amplification (Fig. 3). SCAR primers amplified the characterized regions from genomic DNA under stringent conditions, which makes these markers more specific and dependable as compared to ISSR markers. This suggests sensitivity and specificity of SCAR marker towards *B. thwaitesii.*

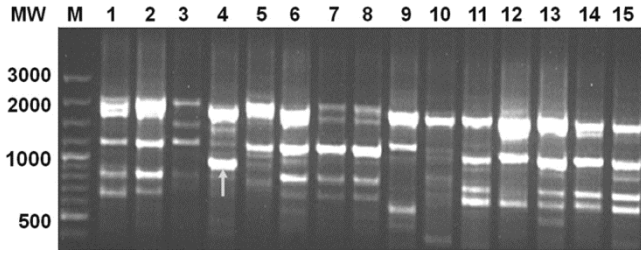

Fig. 1 — ISSR profiles (UBC-857) of Pandanaceae species showing unique bands in *B. thwaitesii* (M: Molecular weight marker, 1: *P. odorifer*, 2: *P. kaida*, 3: *P. unipapillatus*, 4: *B. thwaitesii*, 5: *P. amaryllifolius*, 6: *P. furcatus*, 7: *P. palakkadensis*, 8: *P. mangalorensis*, 9: *P. dubius*, 10: *B. foetida*, 11: *P. emerginatus*, 12: *P. diversus*, 13: *P. nepalensis*, 14: *P. martinianus*, 15: *P. unguifer*).

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GCACCGCATC CAGCTCCGGC CGCCATGGCC GCGGGATTCA CACACACCGA CGCCGTGACA 60
TTAAAATTCT TCTCTTTCCA TTGACGCAAG CAATTGTTAA GTTGTGCTTC GTCACGGGAA 120
CATGTGTGTA CTGACACCCC GAACCCAGCT AGTTCTTCAA CTATGGCACG CCTGACACAG 180
GAGGCCGTTG AGGAAAAGAA CAGACCTCTC TTAGCCACTA TTGGCAAGAA AAGGGAAAAG 240
CAGGCTACTT CATATATGTT TTCATTTCTA ACTTTATCCC AAGAGAAGTA AAATTAATTT 300
CCTGCTGTAT ACTATCTTTG TGTTATTTCT TTGGCTGGAA AATCAATTGA AACTTTTCTG 360
GAACTAACAA ACTTGATTTG ATCTTGTATA TTTACCTTCT ATATTCATAG GACATTGTTT 420
TTTTCCTTCC TTATCATATA TATATTTTTT TAATTTGTTC TATTTGCTAA ACGGCACACT 480
ATTCAGATTC GACAGAAAAA GCTAGAAAGA AGCCATGTTA AAGGGCAGTG AGGAGAAGGA 540
TACCCAATTC CTTTGGAGCC TCCGGTGACT AAAGCGGTGG CCCCATGGAG GGGCCATCTC 600
TTTGCTCTGC TGGTATCGGC AGTGATCTCG ACGTTATCCC CCATCGAAAT CTCCTCTCCA 660
CCTTGTAATA CGGATGTTCT CTTCTGCTCT CATCTCCTAC ATGCACTGGT GCTGTGTAAG 720
ATGGATGTAT GCCCACAGAA GGAGAGCCAT GTGACGTGGC GCTAGCCTAA GCTGCAGTTT 780
GGTCGCCGCC CAATTCCAAG ACGGCAAAGC CAGTGGATGT CCAAGACAGC TGACGCAAGA 840
CGGCAGCTCA ATCTAAAACA CCAACGGATG AATTTTACCT CAAATGACTA ACATACCAGT 900
GTGTGTGTGT GTGTAATCAC TAGTGGCGGC CGCCTGCAGG GTTCGACCAT ATGGGAGAGT 960
CCCAACGCGT TGGATTGTT 979
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Fig. 2 — Sequences of distinctly amplified *B. thwaitesii* ISSR amplicon.

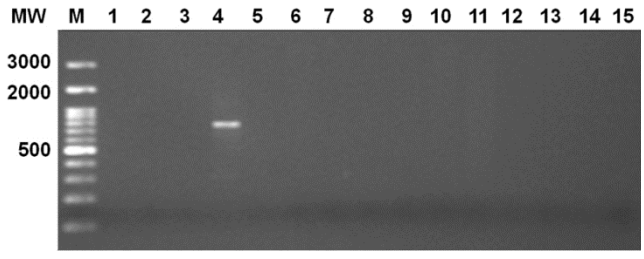

Fig. 3 — Sequence characterised amplified region (SCAR) in *B. thwaitesii* (M: Molecular weight marker, 1: *P. odorifer*, 2: *P. kaida*, 3: *P. unipapillatus*, 4: *B. thwaitesii*, 5: *P. amaryllifolius*, 6: *P. furcatus*, 7: *P. palakkadensis*, 8: *P. manglorensis*, 9: *P. dubius*, 10: *B. foetida*, 11: *P. emerginatus*, 12: *P. diversus*, 13: *P. nepalensis*, 14: *P. martinianus*, 15: *P. unguifer).*

Discussion

Sex-specific SCAR marker have been developed in commercially explored dioecious *P. odorifer* (*P. fascicularis*) for distinguishing male genotype from female genotype, which is useful in perfume industry for selection of male plant in the early vegetative growth phase[12]. Recently, we traced the fragrance potential of some South Indian Pandanaceae species and observed that, male inflorescence of *P. kaida*, *P. palakkadensis* and *B. thwaitesii* recorded comparable fragrance score with *P. odorifer* and holds good potential for commercial cultivation and extraction of essential oil[6]. Because of fragrance potential of *B. thwaitesii* male inflorescences are sold in local markets and extracted for essential oils. In order to distinguish *B. thwaitesii* from other Indian Pandanaceae species, the generated species-specific SCAR marker could be successfully used in identification. In future, for commercial extraction of *B. thwaitesii* in perfume industry, this SCAR marker will be useful to distinguish it from other Indian Pandanaceae species.

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