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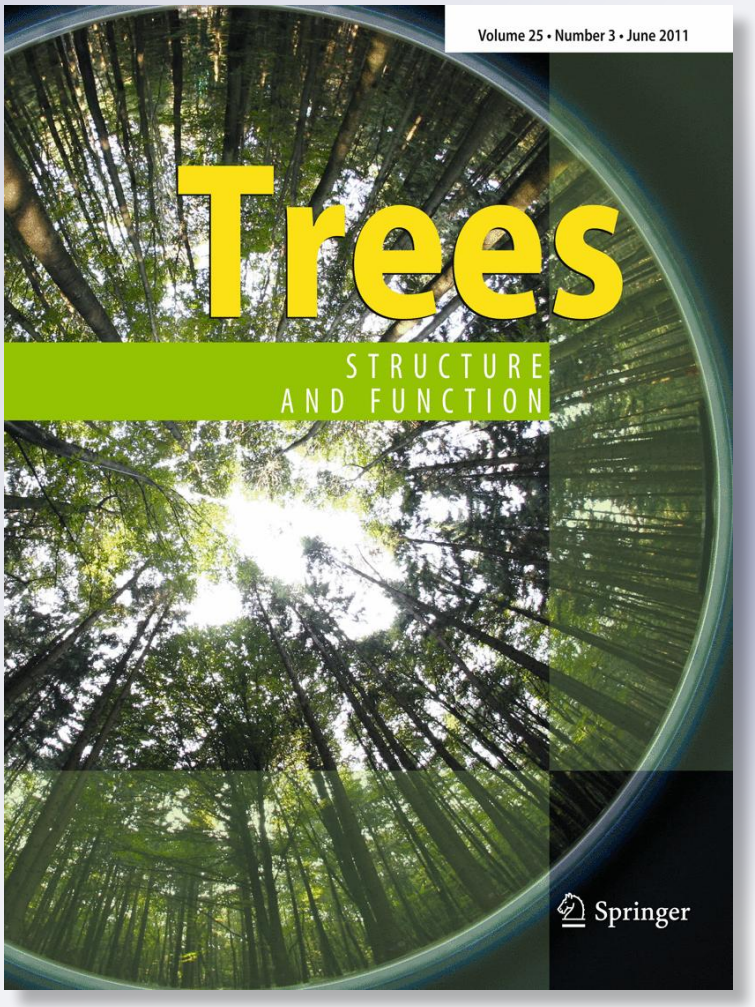
Trees
Structure and Function

ISSN 0931-1890
Volume 25
Number 3

Trees (2011) 25:465-472
DOI 10.1007/s00468-010-0522-x



Springer

Trees (2011) 25:465–472
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ORIGINAL PAPER

Development of SCAR marker in *Casuarina equisetifolia* for species authentication

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Received: 28 May 2010 / Revised: 15 November 2010 / Accepted: 19 November 2010 / Published online: 14 December 2010

Abstract *Casuarina equisetifolia* is one of the most extensively introduced tree species outside its natural range and is a true multipurpose species, providing a range of services and products for industrial and local end users. Natural hybrids of *C. equisetifolia* have been reported and a putative hybrid of *C. junghuhniana* and *C. equisetifolia* is commercially cultivated in Thailand and India. In Taiwan, studies have also revealed that most plants of *Casuarina* are the result of introgressive hybridization involving *C. equisetifolia*, *C. glauca*, and *C. cunninghamiana* causing difficulty in species identification. The present study was aimed at developing DNA markers for the identification of *C. equisetifolia*. Seven ISSR primers were amplified in 120 individuals belonging to three species of *Casuarina* (*C. equisetifolia*, *C. glauca* and *C. junghuhniana*) and two *Allocasuarina* species (*A. littoralis* and *A. huegeliana*). One species-specific amplicon at 650 bp amplified in all individuals of *C. equisetifolia* was cloned, sequenced and primer pairs were developed and designated as IFGTBCE01. The sequence characterized amplified region (SCAR) marker was multiplexed with internal SSR primer pair (positive control) and amplified in ten randomly selected individuals of *C. equisetifolia*, *C. junghuhniana*, *C. glauca*, *A. littoralis* and *A. huegeliana*. The species-specific band amplified only in *C. equisetifolia* at 500 bp. Subsequently, the SCAR marker was validated in 30 individuals collected from 10 provenances belonging to 9 countries and in 10 locally selected clones. The marker was validated in the natural male hybrid of *C. equisetifolia* × *C. junghuhniana* which is widely planted in India for fast growth, wide adaptability and good form. Hence, this marker can be used for accurate and rapid identification of the species during certification and determination of putative hybrids in breeding programs.

Keywords Casuarina · Hybrid validation · ISSR · Multiplexed PCR · Species-specific

Introduction

The family Casuarinaceae consists of 4 genera, including *Allocasuarina* L. Johnson, *Casuarina* L. Johnson, *Ceuthostoma* L. Johnson, *Gymnostoma* L. Johnson and 97 species of monoecious or dioecious shrubs and trees (Wilson and Johnson 1989; Moneur et al. 1997). All the genera grow in tropical climates, except *Casuarina*, which extends to warm temperate regions of Australia, while *Allocasuarina* is distributed mainly in warm to cool temperate regions of Australia (Steane et al. 2003). Among the four genera, the species of *Casuarina* and *Allocasuarina* are commercially cultivated in many tropical and subtropical regions of the world. *Casuarina equisetifolia* is a small to large tree that has a wide natural distribution in subtropical and tropical coastlines from northern Queensland and the Northern Territory in Australia, throughout southern Thailand, Malaysia (Peninsular Malaysia, Sabah and Sarawak), the Philippines, Indonesia, Melanesia and Polynesia (Pinyopusarerk et al. 2004). It is one of the most extensively introduced tree species outside its natural range, especially into southern China, India, Vietnam, East, West and North Africa, Central and South America, the

Communicated by J. Carlson.

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Caribbean and many Middle Eastern countries. Some of the early introductions, such as those to India and Vietnam, date back more than a century (Kondas 1983; Ha and Kha 1996). There are 300,000 ha of *C. equisetifolia* plantations in southern China (Zhong and Bai 1996), 800,000 ha in India (Warrier 2009) and 120,000 ha in Vietnam (Ha and Kha 1996). Casuarinas are cultivated for landscaping, pulp, lumber, medicine, tannin, dye and sand-shifting control in coastal areas (Pan et al. 1996).

The species of *Casuarina* and *Allocasuarina* are taxonomically distinguished based on the color of mature samaras, woody nature of cone bracteoles and number of teeth per whorl (Wilson and Johnson 1989). Among the three *Casuarina* (*C. equisetifolia*, *C. junghuhniana*, *C. glauca*) and two *Allocasuarina* (*A. littoralis*, *A. heugeliana*) species, the characters which differentiated the species included plant height, branch length, internode length and teeth length (Kamalakannan et al. 2006). Natural hybrids of *C. equisetifolia* with *C. junghuhniana*, *C. cunninghamiana* and *C. glauca* have been reported (Ho et al. 2002b) and a putative hybrid of *C. junghuhniana* and *C. equisetifolia* is commercially cultivated in Thailand (Chittachumnonk 1983). In Taiwan, studies have revealed that most plants of *Casuarina* are the result of introgressive hybridization involving *C. equisetifolia*, *C. glauca*, and perhaps *C. cunninghamiana* and are derived from the backcrossing to *C. equisetifolia*, causing difficulty in species identification (Ho et al. 2002b). In India, a natural male hybrid of *C. equisetifolia* × *C. junghuhniana* is widely planted for its superior growth (Jayaraj 2010). Until now, the identification of this species was based on their morphological descriptors which failed to reveal their exact taxonomic affinity since most of the morphological characters show phenotypic plasticity and are influenced by environmental factors. Nevertheless, with the increase in demand for identified species and hybrids in breeding programs, a marker-based identification system has become a critical need.

PCR-based DNA markers are now used by systematists and germplasm managers for species/cultivar identification and validation in trees as reported in eucalypts (Grattapaglia et al. 1992; Balasaravanan et al. 2006), spruce (Khasa and Dancik 1996), *Morus* spp. (Tani et al. 2003), olive (Bautista et al. 2003) and *Larix* sp. (Gros-Louis et al. 2005). They were also used to verify parents in hybridization programs and release of consistent hybrids in *Eucalyptus graniticola* (Rossetto et al. 1997) and spruce (Nkongolo et al. 2003, 2005).

The ambiguity associated with markers like RAPD and ISSR can be overcome by converting them into sequence characterized amplified regions (SCARs) (Paran and Michelmore 1993). Species identification using SCAR makers has been demonstrated in very few woody species like olive (Hernandez et al. 2001), pine (Nkongolo et al. 2002), *Picea* (Nkongolo et al. 2003), bamboo (Das et al. 2005), *Pyrus pyrifolia* and *P. communis* (Lee et al. 2004) and *Hevea* (Venkatachalam et al. 2006).

In casuarinas, there are no reports on identification of species-specific markers and hence the present study was aimed at development of species diagnostic SCAR marker from specific ISSR markers in *C. equisetifolia*, the predominant species used in tropical and subtropical countries for breeding program and its validation in a natural hybrid. This is the first report on generation of SCAR markers in the family Casuarinaceae.

Materials and methods

Plant material

Seeds of three *Casuarina* species (*C. equisetifolia* L., *C. junghuhniana* Miq., and *C. glauca* Sieb. Ex Spreng) and two *Allocasuarina* species (*A. huegeliana* (Miq.) L. Johnson and *A. littoralis* (Salisb.) L. Johnson) were obtained from the Australian Tree Seed Centre, CSIRO, Australia (Table 1). The seedlings were raised in the germplasm bank of Institute of Forest Genetics and Tree Breeding, Coimbatore, India and were used for raising a species trial at Panampally Research Station at Kerala, India.

Table 1 Seed source of *Casuarina* and *Allocasuarina* species used in the ISSR study

Species name	CSIRO seed lot number	No. of parent trees	Locality	Altitude (m)	Latitude (N)	Longitude (E)
C. equisetifolia ssp. *equisetifolia*	19129	4	Lakei/sibur Bako MLAY	40	1°44′	111°29′
C. glauca	15941	9	Burrum Heads QLD	1	25°12′	152°37′
A. huegeliana	15801	4	Sanford Rock WA	380	31°13′	118°46′
A. littoralis	13876	5	Gordan and Chili Cks QLD	80	12°42′	143°20′
C. junghuhniana ssp. *junghuhniana*	19489	10	Kapan Kupang INDO	700	10°13′	123°38′

Table 2 Details of *C. equisetifolia* provenances used for SCAR validation

Country	CSIRO seed lot number	Provenance	Latitude (N)	Longitude (E)	Altitude (m)
China	18268	Daodong State Forest Farm, Hainan	19°58′	110°59′	10
Egypt	18122	Montazah National Park	31°16′	30°05′	13
India	18015	Chandipur, Balasore, Orissa	21°30′	86°54′	2
Kenya	18142	Kilifi	3°38′	39°51′	20
Malaysia	19129	LaKei/sibur Bako	1°44′	111°29′	40
Papua New Guinea (PNG)	18153	Ela Beach	9°05′	147°17′	5
Philippines	18154	Tangalan, Aklan Pt. Panay Island	11°55′	122°23′	30
Thailand	18297	Ban Kam Phuam Ranong	9°21′	98°27′	10
Vietnam (1)	18152	Ninh Chu Binh Thuan	11°33	108°59	2
Vietnam (2)	18128	Non Nuoc Quang Nam, Da Nang	16°06′	108°21′	2

Table 3 List of *C. equisetifolia* clones used for SCAR validation

S. no.	Clone no.	Place of selection	Latitude (N)	Longitude (E)
1	CHCE892002	Chidambaram, Tamil Nadu, India	11°54′	71°41′
2	CHCE890304	Chidambaram, Tamil Nadu, India	11°54′	71°41′
3	CHCE890903	Chidambaram, Tamil Nadu, India	11°54′	71°41′
4	CHCE890905	Chidambaram, Tamil Nadu, India	11°54′	71°41′
5	CHCE892703	Chidambaram, Tamil Nadu, India	11°54′	71°41′
6	CHCE890102	Chidambaram, Tamil Nadu, India	11°54′	71°41′
7	CPCE891501	Chengelpet, Tamil Nadu, India	13°00′	80°11′
8	CPCE890110	Chengelpet, Tamil Nadu, India	13°00′	80°11′
9	CPCE893903	Chengelpet, Tamil Nadu, India	13°00′	80°11′
10	CPCE890401	Chengelpet, Tamil Nadu, India	13°00′	80°11′
11	CPCE891802	Chengelpet, Tamil Nadu, India	13°00′	80°11′
12	CPCE890301	Chengelpet, Tamil Nadu, India	13°00′	80°11′

The validation of species diagnostic marker was conducted in ten selected provenances of *C. equisetifolia* belonging to nine countries and the germplasm was collected from Regional Forest Research Centre, Rajahmundry, Andhra Pradesh, India (Table 2). The provenance trial was established with 100 families drawn from an international collection of 17 provenances and 10 countries with seed source from CSIRO, Australia (Rao et al. 2001). Further, the marker was validated in ten superior performing clones of *C. equisetifolia* selected by the Institute of Forest Genetics and Tree Breeding, Coimbatore, India (Kumar and Gurumurthi 2000). The source of the clonal material was from the populations distributed along the east coast in southern part of Tamil Nadu, India (Table 3).

The SCAR marker was also validated in a natural male hybrid of *C. equisetifolia* × *C. junghuhniana*. The hybrid was introduced in India in 1951 from Thailand and is planted in large scale due to its fast growth, wide adaptability and good form when compared to *C. equisetifolia* (Thirawat 1953). The hybrid is multiplied and marketed in large scale by Crossandra Innovation Centre, Puducherry as 'MIQ-Junghuhniana casuarina', while tissue culture raised propagules are marketed by M/s Growmore Biotech, Hosur. Paper and pulp industries including Andhra Pradesh Paper Mills, Rajahmundry and Tamil Nadu News Prints and Paper Ltd., Karur supply vegetatively propagated hybrids to farmers for planting (Jayaraj 2010).

Three individual collections including 'MIQ-Junghuhniana casuarina' by Crossandra Innovation Centre, Puducherry, India, tissue culture raised plantlet from M/s Growmore Biotech, Hosur, India and collection from Tamil Nadu Forest Department, India were selected for marker validation.

Table 4 List of ISSR primers used for profiling *Casuarina* and *Allocasuarina* species showing number and size range of amplified products

Primer code	Nucleotide sequence 5′–3′	Amplification range (bp)	Total no. of bands	Polymorphic bands
5′ anchored				
$R(CA)_7$	GRTRCYGRTRCACACACACACACA	270–1,636	34	11
$T(GT)_9$	CRTAYGTGTGTGTGTGTGTGTGT	365–1,018	36	10
$TA(CAG)_4$	ARRT YCAGCAGCAGCAG	220–1,710	44	13
$RA(GCT)_6$	AYARAGCTGCTGCTGCTGCT	220–700	28	5
3′ anchored				
$(GA)_8R$	GAGAGAGAGAGAGAGARGY	320–1,035	26	12
UBC810	GAGAGAGAGAGAGAGAT	335–1,225	41	21
UBC842	GAGAGAGAGAGAGAGAYG	330–1,140	32	7
Mean			34.43	11.29
SD			6.53	5.12

DNA isolation and ISSR profiling

Twenty-three to 25 individuals from each species (120 individuals) were randomly collected for DNA isolation for ISSR profiling. Total genomic DNA was extracted from 100 mg of fresh juvenile needles using Qiagen DNeasy plant mini kit (Qiagen, Hilden, Germany) as described by the manufacturer. Extracted DNA was quantified using spectrophotometer and comparing band intensities with known standards of lambda DNA (Bangalore Genei Ltd, India) on 0.8% agarose gels.

Seven ISSR primers were custom synthesized (Sigma-Aldrich, USA) for the study. The primers were selected based on the earlier report by Yasodha et al. (2004) (Table 4). PCR amplifications were performed in a 10 µl reaction volume containing about 30 ng of template DNA, 1.0 µl 10× PCR buffer (Bangalore Genei Ltd, India), 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 100 nM primer and 0.3 U Taq DNA polymerase (Bangalore Genei Ltd, India). PCR amplifications were carried out in a programmable thermal cycler (PTC-200, MJ Research, Inc., USA) with initial denaturation for 3 min at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 50°C, extension for 1 min at 72°C and a final extension for 10 min at 72°C. Amplification products were resolved in a 2% agarose gel with 1× TAE buffer at 70 V for 2 h along with 1 kb ladder (Gibco BRL Ltd, USA) for molecular weight determination. The gel profiles were viewed under UV-transilluminator and documented using Kodak-DC290 digital camera.

ISSR analysis and marker selection

The presence of an ISSR fragment in a particular species population and its absence in all the other species was designated as species diagnostic marker. Further, primer amplifying the specific band was separated in a 4% denaturing polyacrylamide gel and stained with silver nitrate as described by Bassam et al. (1991). The specific amplicons were excised from the acrylamide gel after treating it with 2 M NaOH for 10 min. The gel fragments were subsequently washed and the DNA was extracted in TE buffer. The fragments were re-amplified using the same program as described above, except that the annealing temperature was increased to 54°C.

Cloning and sequencing of specific ISSR fragments

The desired band of C. *equisetifolia* was cloned into pDrive vector (Qiagen, Hilden, Germany) using the manufacturer's protocol. Recombinant clones were selected by blue white screening (Sambrook et al. 1989) and sequenced using ABI Prism 3100 automated DNA sequencer.

Sequence data analysis, designing of SCAR primers and validation

The sequence data was analyzed for the presence of SSR repeat motifs using WebTroll (http://wsmartins.net/websat). Primer pairs were designed using primer 3 software (http://www.genome.wi.mit.edu/ftp/pub/software/primer.3.0), custom synthesized from Sigma-Aldrich, USA and was designated as IFGTBCE01. The marker was multiplexed with an internal positive control of SSR primer pair (CESSR22) (Yasodha et al. 2009; GenBank Accession number AY839233). These primer pairs were amplified in ten randomly selected individuals from all five species using the amplification conditions of 5 min at 94°C, followed by 29 cycles of denaturation for 1 min at 94°C, annealing for 30 s at 62°C, extension for 2 min at 72°C and a final extension for 10 min at 72°C (Table 5). Further, 40 individuals selected from diverse populations

Table 5 Primer pairs used for species diagnosis and multiplexed PCR analysis

SCAR/SSR primer pair	5′–3′ sequence	Repeat motif	Expected size (bp)	T_m (°C)
IFGTBCE01-FP	AAGACGACGGAGAAGCAAAA	$(GA)_6$	500	62
IFGTBCE01-RP	CGAGAACAGTGGAGGAGGTC			
CESSR22-FP	CATTGGTGCAATTCCAAAGA	$(GCT)_4$	219	63
CESSR22-RP	CCAGCTCCAGCTTCAGTACC			

GAGAGAGAGAGAGAGACGAAGATTCGTCACAACCCCTTTTTGCAAGGGACA
AAATGCGTTTTTAAAAACAAGACGAAGAAGAAGAAGCAGAAGAAGAAGAAG
AAGAAGACGACGGAGAAGCAAAA TGGGTCATAATTTGGATGTAGCAAAAGA
TTTACTACTGAAACTAACATTTGTATAAAGTGACAAAAAGCAATTCTCTTTCA
TTTACATTTGGAAGTAACAAATTTCAGGTAAACATATCTTCACTAGCTCTAGA
CCAACAATCTCCACATTCTGTAAATTGTGCAACAGAATGATGAGAGAGAGAG
ACAGAGAGATCATTCCCTAACAAGCAAAAAATTTAATCGAAAAGAATGGATC
ATGTGACGCTGTATATGGAGTATTCACAGAACGTCTTCATCAAGATAGAGTC
CCCTGGTGCTCTCTGTAGAACTATGGTCAGAAACTGTCGAAACAATTGAGCT
GCTGCTGTCAGTCTCACTGTCAAAAGCAAACCTATCAACAACCTAAGTTAGC
AGATAACATCTGGCAAGCCTATAAGAACTCCCTAATCACATCATAAACAGGT
ATTTGACCTCCTCCACTGTTCTCG GTATCGGATGCGGTATTCCCTCCATTAACT
ACCTCTATCCATCTCTCTCTCTCTCTC

Fig. 1 Sequence of species diagnostic ISSR marker in *Casuarina equisetifolia* showing primer sites of ISSR and SCAR marker. *Shadowed area* represents binding site of ISSR primer. *Underlined area* represents binding sites of SCAR markers

(see Table 2) and 3 individuals of the natural hybrid were amplified with the species diagnostic SCAR marker.

Results and discussion

Identification of species-specific ISSR fragments

The 7 ISSR primers produced distinct and polymorphic profiles in all 120 individuals. A total number of 241 scorable PCR products were detected in the size range between 220 and 1,710 bp. Table 4 shows primer-wise details of the amplification products generated by the three *Casuarina* and two *Allocasuarina* species. A maximum of three specific amplicons was identified in *A. littoralis* and a minimum of one was observed in *C. equisetifolia*, *C. glauca*, and *A. huegeliana*. In *A. littoralis*, specific amplicons were observed at 365, 760 and 890 bp using $T(GT)_9$, $TA(CAG)_4$, UBC810 primers, while in *A. huegeliana*, *C. equisetifolia* and *C. glauca*, specific amplicon was documented at 445, 650 and 830 bp using $R(CA)_7$, UBC842 and $T(GT)_9$ primers, respectively. The putative markers were resolved in 4% denaturing PAGE. Only one amplicon at 650 bp amplified by primer UBC842 in *C. equisetifolia* was found to be present in all individuals of the species and was absent in all individuals of the other four species.

Sequence analysis, primer designing and amplification of SCAR markers

The specific band of 650 bp was cloned, sequenced (Fig. 1; GenBank Accession number EI108881). The sequence data were analyzed and the first and last 18 bases of the sequence corresponded to the UBC810 primer sequence and revealed the presence of dinucleotide repeat $(GA)_6$. Primer pairs were designed spanning the microsatellite region and designated as IFGTBCE01 (Table 5). This SCAR marker was multiplexed with the internal positive control of SSR primer pair CESSR22 and amplified in ten individuals of all the five species. The SSR primer pair amplified in all 50 individuals at approximately 220 bp revealing the positive control for PCR. Additionally, all ten individuals of *C. equisetifolia* amplified the species diagnostic band at 500 bp, which was absent in the other four species, highlighting the specificity of the SCAR marker toward species diagnosis (Fig. 2). The marker was validated in 30 individuals from *C. equisetifolia* provenances collected from diverse sources including China (3), Egypt (4), Kenya (2), Malaysia (5), Papua New Guinea (PNG) (2), Philippines (3), Thailand (4), India (3) and Vietnam (4). The marker was also amplified in ten locally selected clones of *C. equisetifolia*. All the 40 individuals showed the amplification of the diagnostic band at 500 bp revealing the species specificity of the SCAR marker (Fig. 3). The marker was further validated in three individuals of a vegetatively propagated natural male hybrid (*C. equisetifolia* × *C. junghuhniana*) and the amplification of the marker at 500 bp ascertained the parentage of the hybrid (Fig. 4).

DNA markers have been used in *Casuarina* to assess the genetic diversity between species (Yasodha et al. 2004; Kamalakannan et al. 2006), within *C. equisetifolia* (Ho et al. 2002a), within *C. junghuhniana* provenances (Ho et al. 2004) and to document intra-specific hybrids in casuarinas (Ho et al. 2002b). Till date, there are no reports

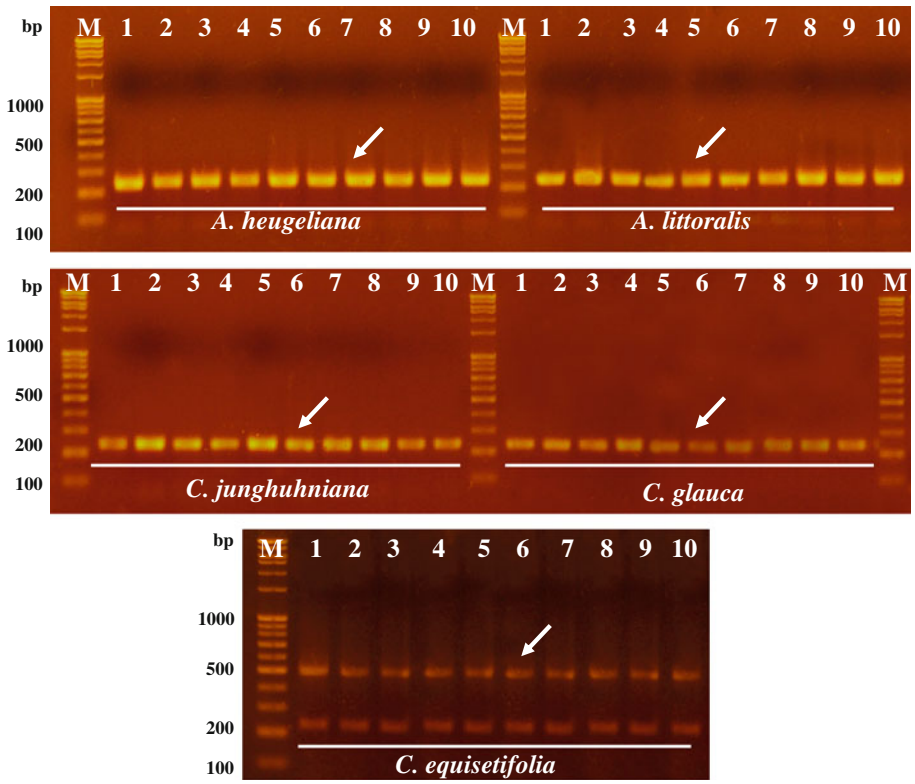

Fig. 2 Multiplexed PCR profile of positive internal CESSR22 and IFGTBCE01 in *Casuarina* and *Allocasuarina* species. *M* 100 bp ladder, *lanes 1–10* indicate randomly selected individuals in each species. *Arrow* at 500 bp indicates species diagnostic marker in *C. equisetifolia* while *arrow* at 220 bp represents the internal positive control amplified with CESSR22

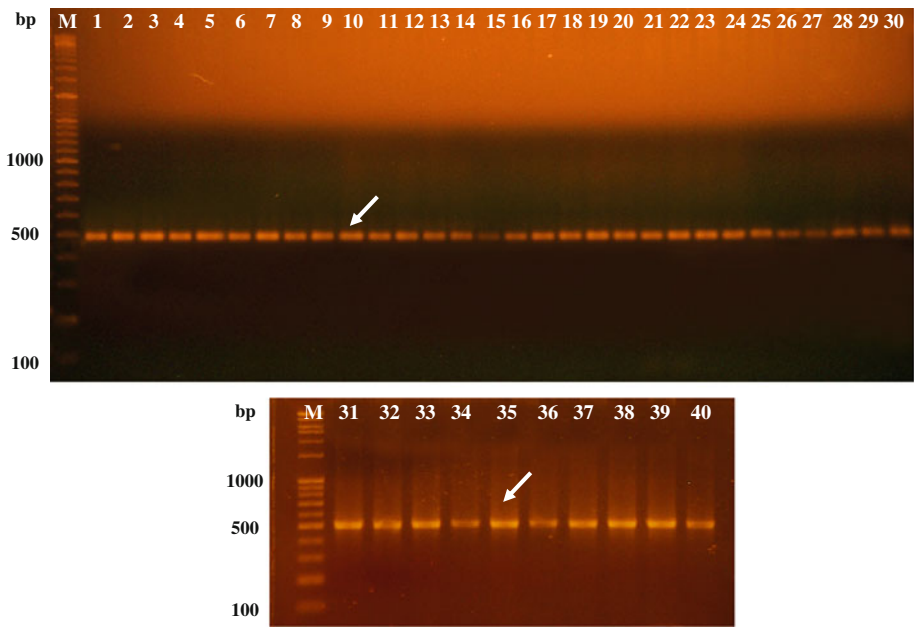

Fig. 3 ISSR–SCAR marker profile of *C. equisetifolia* populations with IFGTBCE01b. *M* 100 bp ladder, *1–4* Thailand, *5–8* Egypt, *9–11* China, *12–14* Philippines, *15*, *16* Kenya, *17*, *18* Papua New Guinea (PNG), *19*, *20* Vietnam (1), *21*, *22* Vietnam (2), *23–25* India, *26–30* Lakei/sibur Bako, Malaysia, *31–40 C. equisetifolia* clones. *Arrow* indicates species diagnostic marker. Details of populations given in Tables 2 and 3

on the development of species diagnostic SCAR markers in this genus. The present study demonstrates the development of species-specific SCAR marker in *C. equisetifolia*. The application of the marker in identification of parentage and confirmation of hybridity was also demonstrated in a natural hybrid of *C. equisetifolia* × *C. junghuhniana*.

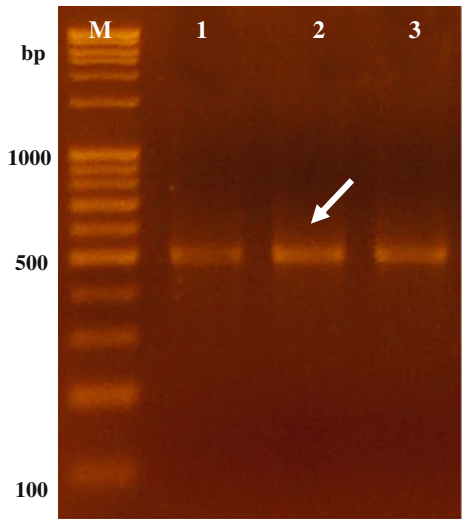

Fig. 4 ISSR–SCAR marker profile of natural male hybrid of *C. equisetifolia* × *C. junghuhniana* with IFGTBCE01b. *M* 100 bp ladder, *1* hybrid (MIQ-Junghuhniana casuarina' from Crossandra Innovation Centre), *2* hybrid (tissue culture raised plantlet from M/s Growmore), 3 hybrid (clone from Tamil Nadu Forest Department). *Arrow* indicates species-diagnostic marker

Hence, the SCAR marker developed in the present study can be used for accurate and rapid identification of species for certification and determination of putative hybrids during selection and hybridization programs of casuarinas.

Acknowledgments This research was funded by the Department of Biotechnology, Ministry of Science and Technology, Government of India.

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