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Genetic analyses of Casuarinas using ISSR and FISSR markers

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Abstract

Inter simple sequence repeat polymerase chain reaction (ISSR-PCR) was used for the genetic analysis of the six species of *Allocasuarina*, five species of *Casuarina* and 12 superior performing selections of *C. equisetifolia* L. We also fingerprinted *C. equisetifolia* L. selections using Fluorescent-ISSR-PCR (FISSR-PCR), an improvised ISSR-PCR assay. The ISSR analysis provided information on the frequency of various simple sequence repeats in the casuarina genome. The di-nucleotide repeats were more common, among which $(CA)_n$ and its complementary nucleotide $(GT)_n$ repeat motifs amplified relatively higher number of bands with an average of 6.0 ± 3.5 and 6.3 ± 1.8 respectively. Eleven species of casuarinas were amplified with 10 primers anchored either at 5′ or 3′ end. A total of 253 PCR products were obtained and all were polymorphic, out of which 48 were specific to *Allocasuarina* and 36 were specific to *Casuarina* genus. Genetic similarity among the species was 0.251. A UPGMA dendrogram grouped all the *Casuarina* species together. The 12 superior performing selections of *C. equisetifolia* L. produced 57 polymorphic ISSR markers while the FISSR assay revealed 105 polymorphic markers. The primer $CRR(ATT)_4$ distinguished all the selections. DNA profiles obtained with ISSR and FISSR assays would serve as a reference library for the establishment of clonal identity in casuarinas.

Introduction

The members of the family *Casuarinaceae* are commonly known as casuarinas. They are monoecious or dioecious trees or shrubs, having unique needle like branchlets with many articles. Currently, casuarinas are grouped under four genera (Wilson & Johnson, 1989), which encompass over 90 species (Moncur, Boland & Harbard, 1997). Among the four genera, the species of *Casuarina* L. Johnson and *Allocasuarina* L. Johnson are commercially cultivated in many tropical and subtropical regions of the world while the other two genera *Gymnostoma* L. Johnson and *Ceuthostoma* L. Johnson occur as wild species only.

Casuarina ($2n = 18$) comprises about 17 species distributed throughout Southeast Asia, and Australia (Pinyopusarerk & House, 1993) whereas *Allocasuarina* consisting of 59 species are endemic to Australia (Wilson & Johnson, 1989). They are considered to be the nearest relatives of the genus *Casuarina* since they share many common morphological features. The basic chromosome number *of Allocasuarina* varies from $n = 10$ to $n = 14$. Polyploidy, particularly tetraploidy is known to be prevalent in some species like *A. luehmanii*

($2n = 56$) and *A. littoralis* ($2n = 44$). Phylogenetic and taxonomic relationships within the species of casuarinas have been studied using the size and number of chromosomes, pattern of geographical distribution (Barlow, 1959, 1983) and diversification in the morphological characteristics (Wilson & Johnson, 1989) to distinguish the members *of Allocasuarina* and *Casuarina*. Recently, *rbc*L and plastid *mat*K sequences were used for the genetic analysis of *Casuarinaceae* (Sogo et al., 2001) and the study was further extended to decipher the phylogeny of 76 species demonstrating the monophyly of the four genera and examining the relationships within the family (Steane, Wilson & Hill, 2003).

Although about 15 species of *Casuarina* and *Allocasuarina* are recognized for multiple utilization, only *C. equisetifolia* L. is cultivated widely in many countries (Doran & Hall, 1983; Pinyopusarerk & House, 1993) accounting for about 1.4% of tree plantations of the casuarinas (FAO, 1995). This commercial and silvicultural importance of *C. equisetifolia* L. has led to the establishment of multinational provenance trials co-ordinated by CSIRO, Australia (Pinyopusarerk et al., 1996). The species is propagated by cladode cuttings (Gurumurthi & Bhandari, 1988) and yield gain is achieved through clonal plantations using genetically divergent and productive clones (Ahuja & Libby, 1993). In India, major effort is directed towards production of improved planting stocks through selection of superior performers from plantations/provenances and their vegetative propagation. Additionally, seed orchard raised with selected clones can provide good quality seeds for plantations if vegetative propagules fall short of demand. The optimal utilisation of diversity requires genetic charaterisation of the stocks and identification of the selected clones in the early stage.

Morphological characters have been used to estimate genetic divergence of clonal selections of *C. equisetifolia* L. (Kumar & Gurumurthi, 2000). Genetic variation at the population level has been studied by using RAPD markers in *C. equisetifolia* L. (Ho et al., 2002). RAPD variation in casuarina of Taiwan has revealed that most plants in Taiwan are closely related but not typical *C. equisetifolia* L. indicating introgressive hybridisation among *C. equisetifolia*, *C. glauca* and *C. cunninghamiana* (Ho, Yang & Hsiao, 2002).

Our goal in the present study is to generate a reliable DNA based marker system for the characterisation of *Casuarinaceae* in general and *C. equisetifolia* L. in particular. Among many different types of DNA markers, the markers based on microsatellites or simple sequence repeats (SSRs) provide a co-dominant, highly reproducible and genetically informative marker system (Tautz, 1989; Weber & May, 1989; Zietkiewicz, Rafalski & Labuda, 1994; Broun & Tanksley, 1996). However, as of today the information on SSR loci is not available in *Casuarinaceae*. An alternative method, the Inter Simple sequence Repeat-PCR (ISSR-PCR), which takes advantage of the ubiquitously distributed SSRs in the eukaryotic genomes, provides an opportunity to obtain highly reproducible markers without prior knowledge of the DNA sequence, for various genetic analyses.

ISSR-PCR has been used widely in plants for the analysis of genetic relationships between and within species (Salimath et al., 1995; Tsumura, Ohba & Strauss, 1996; Fang, Krueger & Roose, 1998; Ge & Sun, 1999; Ajibade, Weeden & Chite, 2000; Chapman, Parh & Oraguzie, 2000; Huang & Sun, 2000; Martin & Sanchez-Yelamo, 2000), assessment of hybridisation in natural populations (Wolfe, Xiang & Kephart, 1998) and germplasm analysis (Proven, Powell & Waugh, 1996; Gilbert et al., 1999). Further, ISSR-PCR is useful in fingerprinting and characterisation of accessions (Fang et al., 1997; Moreno, Martin & Ortiz, 1998; Blair, Panaud & McCouch, 1999; Charters & Wilkinson, 2000) and identification of cultivars and varieties (Fang & Roose, 1997; Prevost & Wilkinson, 1999; Zavodna et al., 2000; Kumar et al., 2001). Recently, Nagaraju et al., (2002a, b) have illustrated the power of an improved method of ISSR-PCR called fluorescent ISSR-PCR (FISSR-PCR) in revealing differences among closely related genotypes. The advantages of this technique over ISSR-PCR include increased number of bands per primer, requirement of very low quantity (2–5 ng) of template DNA and high throughput analysis.

The present work was taken up with an objective of establishing an efficient and reproducible marker system based on ISSR-PCR for the identification of clonal selections of *Casuarina equisetifolia*. We also attempted to find out genetic relationships among a few species of *Allocasuarina* and *Casuarina* with ISSR-PCR markers.

Materials and methods

Plant material and DNA extraction

Six species of the genus *Allocasuarina* and five species of the genus *Casuarina* were used in the present study (Table 1). Seeds (bulked) were obtained from Australian Tree Seed Centre, CSIRO, Australia and the location of the seed samples collected are shown in Figure 1. Seeds were germinated on sterilised sand and about 20 day old, 20 individuals per species were pooled for DNA extraction.

The *Casuarina equisetifolia* trees were selected from two different plantations raised at Chidambaram (11°54″N, 71°41″E) and Chengalpet (13°00″N, 80°11″E). Among 55 candidate plus tree selections raised as hedges, 12 superior performers were selected based on their silvicultural characteristics, viz., height, diameter at breast height, crown architecture and main bole volume and have been used extensively for establishing commercial clonal plantations (Table 2). These 12 selections (called 'clones' henceforth, solely to indicate the mode of propagation from the selected plants) were chosen for DNA fingerprinting.

Fresh, growing tips of needles of the clones were used for DNA extraction. The plant materials were ground to powder in sterilised pestle and mortar in the presence of liquid Nitrogen and the DNA was extracted as described previously (Doyle & Doyle, 1990). DNA was purified and quantified according to standard procedures.

PCR analysis

In a preliminary study, a total of 37 primers (10 primers of University of British Columbia and 27 in house designed and synthesised at Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India) were tested (20 on two species of *Allocasuarina* – *A. decaisneana* and *A. dielsiana*; 37 on two clones of *C. equisetifolia* L. – CHCE890903 and CPCE890301) (Table 3). Based on the number of polymorphic bands produced, 10 and 7 primers

Table 1. Details of seed sources and basic chromosome number of *Allocasuarina* and *Casuarina* species used for ISSR studies

S. no.	Name of the species	Chromosome number (*n*)	CSIRO seed lot number	Origin	Number of trees used for bulking seeds
1	*Allocasuarina decaisneana* (F. Muell.) L. Johnson	14	17364	Ooraminna NT	14
2	*Allocasuarina dielsiana* (C. Gardner) L. Johnson	14	13215	Rothsay townsite WA	5
3	*Allocasuarina Huegeliana* (Miq.) L. Johnson	13	15801	Sanford Rock WA	4
4	*Allocasuarina littoralis* (Salisb.) L. Johnson	11	13876	Gordan and Chilicks QLD	5
5	*Allocasuarina luehmannii* (R. Baker) L. Johnson	14	13880	Mt. Molloy Mareeba QLD	30
6	*Allocasuarina torulosa* (Aiton) L. Johnson	12	13377	Mt. Lewis QLD	10
7	*Casuarina cristata* Miq.	9	17757	West of Dalby Moonie Hwy QLD	4
8	*Casuarina cunninghamiana* Miq.	9	13513	36 km NE of Oasis QLD	10
9	*Casuarina equisetifolia* L.	9	19129	Lakei/sibur Bako, Malaysia	4
10	*Casuarina glauca* Sieb. Ex Spreng.	9	15941	Burrum Heads QLD	9
11	*Casuarina obesa* Miq.	9	15796	Murchison River WA	10

Figure 1. Location of collection of *Allocasuarina* and *Casuarina* species used in the study. (The numbers represent the order of species given in Table 1.)

Table 2. List of clones of *Casuarina equisetifolia* used for ISSR studies

S. no	Clone no	Sex[a]	Height (cm)	DGL[b] (cm)	DBH[c] (cm)	MBV[d] $(cm)^3$	FV[e] $(cm)^3$	Pole value
1	CHCE892002	M	1574	8.73	6.69	47,084	6514	32,948
2	CHCE890304	M	1376	9.01	6.69	43,844	6785	38,545
3	CHCE890903	M	1650	10.62	8.55	73,042	9995	60,682
4	CHCE890905	M	1450	8.52	5.57	41,313	5618	31,384
5	CHCE892703	F	1650	7.57	6.22	37,112	5170	24,919
6	CHCE890102	F	1340	8.69	6.77	39,718	6535	26,170
7	CPCE891501	M	1574	8.81	6.85	47,950	6701	31,905
8	CPCE890110	M	1425	8.40	7.16	39,465	6544	30,549
9	CPCE893903	M	1350	7.01	6.04	26,038	4586	21,374
10	CPCE890401	M	1340	7.26	5.88	27,722	4680	18,350
11	CPCE891802	F	1340	7.72	7.06	31,346	5868	23,355
12	CPCE890301	F	1300	7.36	5.75	27,640	4700	21,166

[a] M = male, F = female; [b] diameter at ground level; [c] diameter at breast height; [d] main bole volume; [e] frustum volume.

were selected for analysing the 11 species of casuarinas and 12 clones of *C. equisetifolia* L., respectively. The selected ISSR primers, which, include di-, tri-, and tetra-nucleotide repeats used for the analysis of species of *Casuarina* and *Allocasuarina* and the clones of *C. equisetifolia* L. are shown in Tables 4 and 5, respectively. ISSR-PCR amplifications were carried out in the reaction mixtures of 10 µl containing 1x PCR buffer (MBI Fermentas), 0.1 mM each of dCTP, dGTP, dTTP and dATP, 2.5 mM $MgCl_2$, 0.8 µM primer, 1.0 unit of Taq DNA polymerase (MBI Fermentas) and 30 ng of template DNA. All the PCR reactions were carried out in PE 9600 Thermal Cycler (Perkin Elmer Corp., USA) using the following reaction conditions: 94°C for 3 min. initial dena-

Table 3. List of ISSR Primers used for preliminary screening

Primer code	Primer sequence 5′–3′	No. of bands amplified		
		Clones	Species	Average
5′ anchored				
$R(CA)_7$	GRT RCY GRT R $(CA)_7$	10	7	8.5
$R(TG)_7$	RYA CRY RCA R $(TG)_7$	3	5	4.0
$Y(TG)_7$	YAY GYA CAY$(TG)_7$ T	0	5	2.5
$T(GT)_9$	CRT AY $(GT)_9$	8	7	7.5
$T(GA)_8$	YGY RAY $(GA)_8$	3	8	5.5
$C(GA)_7$	RAY RAT AY $(GA)_7$	4	9	6.5
$(CA)_7C$	GYT ACT GYT $(CA)_7C$	0	7	3.5
$G(ATT)_4$	YCY RRG $(ATT)_4$	6	ND	ND
$AT(ATT)_4$	RYGAT $(ATT)_4$	4	ND	ND
$CRR(ATT)_4$	AGC RR$(ATT)_4$	4	5	4.5
$TC(ATT)_4$	GAR TY $(ATT)_4$	3	ND	ND
$T3(ATT)_4$	RA TYT $(ATT)_4$	1	ND	ND
$GA(ATT)_4$	AGY GR $(ATT)_4$	3	ND	ND
$AA(AAT)_4$	TR AAR $(AAT)_4$	2	ND	ND
$CT(AAT)_4$	RYR CY $(AAT)_4$	0	ND	ND
$A3(AAT)_4$	ACR ARA $(AAT)_4$	0	ND	ND
$TA(CAG)_4$	ARR TY $(CAG)_4$	4	9	6.5
$RA(GCT)_6$	AYA RA $(GCT)_6$	5	10	7.5
$Y(ACC)_7A$	YYR AY $(ACC)_7A$	6	6	6.0
$(CTT)_7$		0	ND	ND
$(CAC)_6$		0	0	0
$(AGC)_{10}$		0	0	0
$Y(GCC)_4$	TGAGY$(GCC)_4$	9	7	8.0
UBC861	$(ACC)_6$	3	ND	ND
UBC862	$(AGC)_6$	2	ND	ND
UBC873	$(GACA)_4$	6	ND	ND
$(GACA)_4$		6	8	7.0
$(GATA)_4$		0	0	0
3′ anchored				
$(GA)_8C$	$(GA)_8$RGY	7	4	5.5
$(GT)_8R$	(GT) $_8$RYR Y	6	4	5.0
UBC807	$(AG)_8T$	4	ND	ND
UBC809	$(AG)_8G$	3	ND	ND
UBC810	$(GA)_8T$	8	5	6.5
UBC811	$(GA)_8C$	6	ND	ND
UBC812	$(GA)_8A$	0	ND	ND
UBC842	$(GA)_8YG$	6	6	6.0
UBC881	$(GGGGT)_4$	0	ND	ND

ND: not determined.

turation and 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min. followed by final extension of 72°C for 10 min. The PCR products were analysed in 2.0% agarose gel containing 0.5 μg/ml ethidium bromides in 0.5X TBE buffer and run in same buffer at 100 V for 3 h. The gels were viewed

Table 4. List of ISSR primers used for the genetic analyses of *Allocasuarina* and *Casuarina* species showing number and size range of amplified bands resulted from each primer

Primer code	No. of polymorphic bands	No. of bands from *Allocasuarina* species	No. of bands from *Casuarina* species	Amplification range (bp)
5′ anchored				
$R(CA)_7$	22	13	20	250–1150
$Y(TG)_7$	17	16	10	200–1100
TA $(CAG)_4$	20	14	15	320–1100
$CRR(ATT)_4$	14	11	6	350–2000
RA $(GCT)_6$	36	28	14	190–1200
$(GACA)_4$	26	23	15	800–4000
3′ anchored				
$(GT)_8R$	23	21	13	190–1500
$(GA)_8C$	26	19	15	210-650
UBC810	36	23	25	900–4000
UBC842	33	28	17	950–3000

and photographed over UV light in a BioRad Geldoc system.

FISSR-PCR amplifications were performed on the DNA from the clones of *C. equisetifolia* L. with the selected primers (Table 5). DNA amplifications were set up in 5 µl volume containing 15 ng genomic DNA, 1x PCR buffer, 2.5 mM $MgCl_2$, 0.1 mM of each dCTP, dGTP, dTTP and dATP, 0.8 µM primer, 0.4 µM fluorescent dUTP (TAMRA, Perkin Elmer), 0.5 unit of Taq DNA Polymerase (MBI Fermentas). The amplification conditions were same as in ISSR-PCR reactions and the products were run on 5% polyacrylamide gel with 7 M urea, on an ABI automated sequencer at a constant voltage of 3000 kV for 7 h.

Data analysis

The ISSR-PCR bands obtained from the species of casuarinas were scored for the presence (1) or absence (0). A pair-wise similarity matrix was constructed using the Dice similarity coefficient (Sneath & Sokal, 1973). The relationship between the species was displayed as a dendrogram, constructed using NTSYS-pc software (Rohlf, 1992) based on Unweighed Pair Group Method using Arithmetic averages (UPGMA).

Table 5. ISSR-PCR and FISSR-PCR analysis showing number and size range of bands with 12 clones of *C. equisetifolia* L

Primer code	No. of bands amplified		No. of polymorphic bands		Amplification range (bp)	
	ISSR	FISSR	ISSR	FISSR	ISSR	FISSR
5′ anchored						
$R(CA)_7$	13	16	7	15	200–1000	320–900
$Y(TG)_7$	8	25	8	22	375–1000	250–690
$CRR(ATT)_4$	20	25	15	20	150–1020	150–1200
$Y(GCC)_4$	13	24	12	18	250–800	250–1300
$(GACA)_4$	9	13	6	12	275–780	200–1300
3′ anchored						
UBC810	13	11	7	8	575–2500	880–1500
$(GT)_8R$	8	17	2	10	340–1000	310–1000

Results and discussion

Evaluation of ISSR primers

Based on the reproducibility and scorable nature of the bands, six di-, three tri- and one tetra-nucleotide repeat primers were selected for the analysis of 11 species of casuarinas and four di-, two tri- and one tetra-nucleotide repeat based primers were selected for fingerprinting of *C. equisetifolia* L. clones. The reproducibility of ISSR-PCR amplification pattern was tested with two different DNA polymerase enzymes (MBI Fermentas and Ampli Taq DNA polymerase) on two thermal cyclers (Perkin Elmer Corp., USA and MJ Research Inc., USA). All the runs gave identical amplification profiles (data not shown). Further, the reproducibility of ISSR markers on the DNA from vegetatively propagated material was tested by using five ramets of the clone CPCE890110 and no variation was found in the amplification profile (data not shown).

Distribution of microsatellite motifs in casuarina genome

The frequency and distribution of different microsatellite motifs in the casuarina genome were analysed using 37 primers on 2 clonal isolates, CHCE890903 and CPCE890301f and 20 primers on two species, *A. decaisneana* and *A. dielsiana*. Such information could be useful in designing the experiments for microsatellite capture in casuarinas. The primers comprised of 17 different SSR motifs (Table 3). The average number of bands produced by 5′ anchored primers was 4.07 ± 2.0 and 3.77 ± 2.5 in case of 3′ anchored primers. The di-nucleotide repeat motif based primers revealed relatively higher number of bands (4.85 ± 2.15) than tri-nucleotide (3.5 ± 2.8) and tetra-nucleotide repeat motifs (3.3 ± 3.8). The lone penta-nucleotide repeat primer (GGGGT) did not show any amplification. Among the di-nucleotide repeats, the $(CA)_n$ and its complementary nucleotide $(GT)_n$ repeat motifs amplified more number of bands with an average of 6.0 ± 3.5 and 6.3 ± 1.8 respectively. Amplification with the primers based on $(GA)_n$ repeats produced 4.6 ± 2.3 bands. The relative abundance of di-nucleotide repeats in casuarina appears to be similar to *Morns* species (Awasthi et al., 2004) and other tropical trees (Condit & Hubbell, 1991), where $(CA)_n$ repeats have been found to be more common than $(GA)_n$.

Genetic analysis of species

The amplification profiles obtained with the ten ISSR primers for the six species of *Allocasuarina* and five species of *Casuarina* are shown in Table 4. A total number of 253 scorable PCR products was detected in the size range of 190–4000 bp. The mean number of bands produced per species was 23. Among the 11 species of casuarinas analysed, all the 253 bands were polymorphic of which 112 bands (44%) were found to be species specific. A total of 150 bands (average of 30 per species) were detected in *Casuarina* species of which 57 bands (36.0%) were genus-specific. One DNA fragment of size approximately 320 bp (primer $TA(CAG)_4$) was present in all the species of *Allocasuarina*. Three DNA fragments of size approximately 1550 bp (primer UBC842), 625 bp (primer $(CA)_7$) and 320 bp (primer $RA(GCT)_4$) were present in all the species of *Casuarina* analysed. Minimum number of bands (42) was observed in *A. torulosa* and maximum number of bands (63) was observed in *A. dielsiana* and *C. equisetifolia* L. The ISSR-PCR profile obtained with 11 species of casuarinas is shown in Figure 2. The pair-wise similarity coefficient ranged between 0.113 (*A. littoralis*/*A. huegelians*) and 0.571 (*C. cristata*/*C. cunninghamiand*). The average genetic similarity among the species was 0.251 ± 0.085. The average genetic similarity among only *Casuarina* species was 0.366 ± 0.089. The dendrogram in Figure 3 illustrates the ability of the ISSR markers in grouping *Casuarinaceae* species. The clearly dendrogram separated the *Allocasuarina* and *Casuarina* genera. *Allocasuarina* species were distributed in two groups (Al and A2), and all the *Casuarina* species were grouped together (C). Similar clustering of *Casuarina* species was obtained by the investigation based on chloroplast *mat*K sequences (Steane et al., 2003).

Identification in C. equisetifolia *L. clones*

ISSR-PCR of DNA from 12 clones of *C. equisetifolia* L. with seven primers produced a total of 84 bands of which 57 bands (68%) were polymorphic (Table 5). The minimum and maximum number of bands observed were 8 (primer $(GT)_8R$

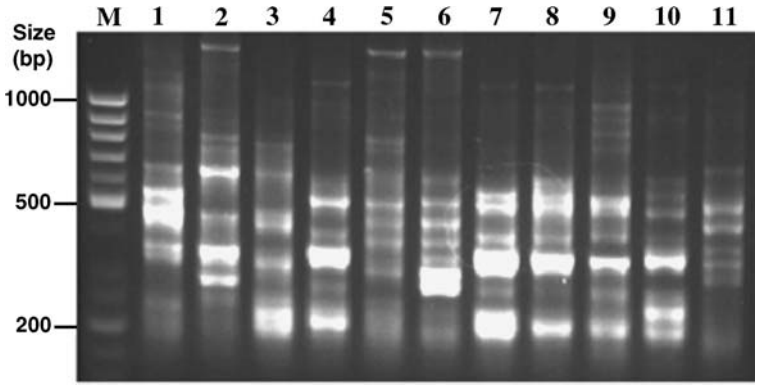

Figure 2. ISSR banding profiles obtained on 2% agarose gel for the 6 *Allocasuarina* and 5 *Casuarina* species with the primer $RA(GCT)_6$. (The numbers represent the order of species given in Table 1) M = Molecular size marker (100 bp ladder, MBI Fermentas).

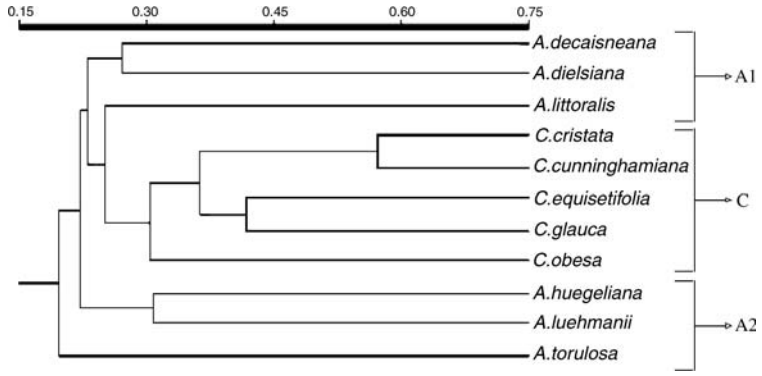

Figure 3. UPGMA dendrogram based on the Dice similarity coefficient illustrating the relationships among the species of *Allocasuarina* and *Casuarina*.

and $Y(TG)_7$) and 20 (primer $(CRR(ATT)_4)$) with an average of 13.6 ± 4.2 per primer. The amplification products ranged in size from 150 bp to 2500 bp. Figure 4 shows the DNA profile generated across the 12 clones using the primer CRR $(ATT)_4$. The average number of bands (12.6 ± 4.7

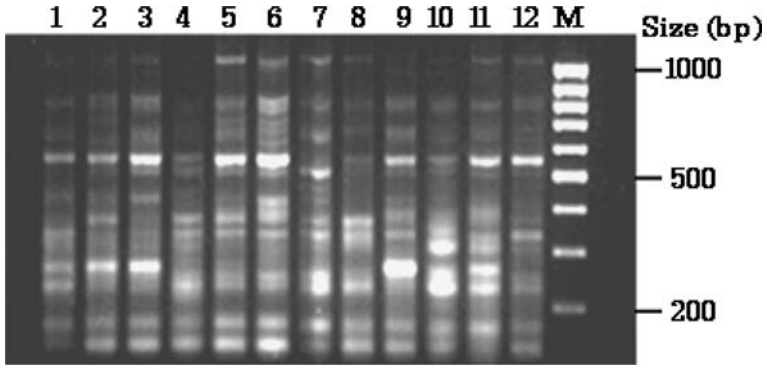

Figure 4. Fingerprints of *C. equisetifolia* L. clones generated by ISSR-PCR on 2% agarose gel using the primer $CRR(ATT)_4$. M = Molecular size marker (100 bp ladder, MBI Fermentas).

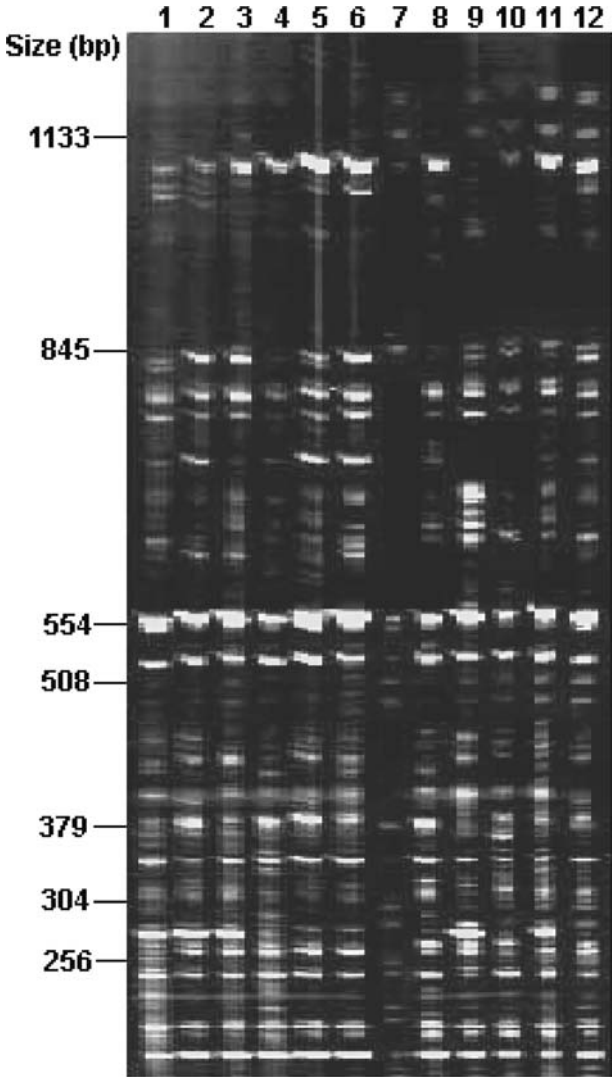

Figure 5. Fingerprints of *C. equisetifolia* L. clones generated by FISSR-PCR on 5% polyacrylamide gel using ABI automated sequencer with the primers $CRR(ATT)_4$. (The numbers represent the order of species given in Table 2.)

per primer) produced by the 5′ anchored primers was higher than the 3′ anchored primers (10.5 ± 3.5). The level of polymorphism was 76% with 5′ anchored primers and 43% with 3′ anchored primers. The di-, tri- and tetra-nucleotide repeat based primers produced 57%, 52% and 67% polymorphisms respectively.

In order to enhance the sensitivity of detection of polymorphisms among clones of *C. equisetifolia* L., we used fluorescent ISSR-PCR (FISSR-PCR) for genotyping. Genomic DNA of the 12 clones was amplified with 7 primers (Table 5). A total of 131 bands were amplified, of which 105 bands (80%) were polymorphic, whereas, in ISSR-PCR the same primers revealed 68% polymorphisms. The mean number of bands produced per primer was 19.0. The minimum and maximum number of bands produced were 11.0 (UBC810) and 25.0 ($Y(TG)_7$ and $CRR(ATT)_4$) respectively. The size of PCR products ranged from 150 bp to 1500 bp. The primer UBC810 produced 11 bands in the size range of 880–1500 bp and clear separation of bands was lacking, where as, on agarose gel the same primer showed 13 bands with clear separation. The amplification size range of this primer in ISSR-PCR was high (575–2500 bp) which could

Table 6. Primers and base pair length of ISSR-PCR and FISSR-PCR generated markers for 12 clones of *C. equisetifolia* L

Primer code	ISSR markers (bp size approx)	Clones identified[a]	FISSR markers (bp size approx.)	Clones dentified[a]
5′ anchored				
$R(CA)_7$	1000, 900, 790	1, 2, 7, 11	680, 620, 430, 350, 320	3, 5, 6, 7, 9, 10, 11, 12
$Y(TG)_7$	780, 750, 700, 640, 340, 275	1, 3, 8, 10, 12	650, 600, 580, 490, 425	1, 6, 7, 9, 10, 12
$CRR(ATT)_4$	615, 430, 380, 300, 275, 250	1–12	700, 680, 620, 555, 420, 285	1–12
$Y(GCC)_4$	360	nil	750, 520, 440, 280	1, 3, 4, 8, 10, 11, 12
$(GACA)_4$	600, 650	nil	1000, 900, 880, 650	1, 3, 4, 6, 9
3′ anchored				
UBC810	1200, 875, 800, 775	1, 2, 5, 10	1200, 1100, 880	3, 5
$(GT)_8R$	900,1000	12	1000, 660, 310	1, 3, 5, 12

[a] See Table 2 for clone number codes.

not be resolved on polyacrylamide gel. Although the primer $Y(GCC)_7$ amplified DNA fragments in the size range of 250–1300 bp, most of them were in the range of 275–560 bp with clear separation. The pattern of amplification products of $CRR(ATT)_4$ is shown in Figure 5.

For fingerprinting purposes, the ISSR and FISSR bands, which were polymorphic for more than four clones were selected and their sizes are given in Table 6. Amplification profile consisting of fourteen polymorphic markers produced by the individual primer $CRR(ATT)_4$ was sufficient to identify all the twelve clones in both ISSR and FISSR assays (Figure 5). These results demonstrated that ISSR assays could generate ideal markers for fingerprinting. It is thus possible to establish a fingerprint reference library of ISSR and FISSR profile of the 12 clones of *C. equisetifolia* L. Such information will be of use in identification and verification of genotypes in clonal plantations and clonal seed orchards. Since the cost of maintaining clones without identity is very high and mislabeling of clones in plantation is not uncommon (Wheeler & Jech, 1992; Keil & Griffin, 1994; Van de Yen & McNicol, 1995; Scheepers, Eloy & Briquet, 1997) the use of DNA based methods for clonal verification will be very useful. No sex specific marker could be identified in our study, though the population from which the samples were collected showed near 1:1 male female ratio with 2–3 percent monoecious trees.

The advantages of ISSR markers are well documented in agronomic species (Godwin, Aitken & Smith, 1997). Genome analysis in woody perennials using ISSR has also been shown to be possible in Douglas fir and Sugi (Tsumura et al., 1996), *Citrus* (Fang & Roose, 1997; Fang et al., 1997, 1998), *Cocoa* (Charters & Wilkinson, 2000), *Larix* (Arcade et al., 2000) and *Morus* (Awasthi et al., 2004). In fingerprinting applications, the molecular method employed should be inexpensive, technically simple yet reliable. In casuarinas, isolation of quality DNA is difficult. Therefore the methodology should require very low quantity (2–5 ng) of template DNA but able to generate higher level of polymorphism. The present study illustrates the advantages of the FISSR-PCR system for the fingerprinting of *C. equisetifolia* clones generating higher number of bands per assay using very little quantity of template DNA.

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