Characterization and Identification of Four Species of Casuarina and their Interspecific Hybrids using EST-SSR Markers

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Abstract
Casuarina species have significant socio-economic importance throughout the tropics and sub tropics by providing fuelwood, pulpwood and environmental services like shelterbelts and windbreaks. In the recent years interspecific hybrids have helped in substantially improving the productivity of Casuarina plantations in India. The objective of the present study was to characterize four species of Casuarina (C. cunninghamiana, C. cristata, C. equisetifolia and C. junghuhniana) and validate their interspecific hybrids using EST-SSR markers. The PCR product size ranged from 233 to 360 bp (mean: 290 bp) and the four primers showed 11 to 20 alleles (mean: 15). The mean values for observed (Ho) and expected (He) heterozygoisty were 0.451 and 0.879 respectively. The polymorphic information content across the primers was from 0.744 to 0928 (mean: 0.85).

The dendrogram obtained from UPMGA analysis of EST-SSR data was able to clearly distinguish the pure species and hybrid individuals and the uniqueness of each individual was also established. Four major clusters were observed one each for C. equisetifolia and C. junghuhniana pure species, hybrids between these two species, C. cunninghamianaan its progeny and C. cristata and its progeny. EST-SSR markers confirmed the hybrid nature of C. equisetifolia x, C. junghuhniana clones and the absence of natural hybridization in C. cunninghamiana and C. cristata trees under open-pollinated conditions. They can be effective in early screening of putative interspecific hybrids from open-pollination and also validate the hybrids produced through control pollination at nursery stage for subsequent field testing.

Keywords: Control pollination, Heterozygoisty, Interspecific, SSR markers and Progeny.

Introduction

Casuarinas are multipurpose tree species belonging to the family Casuarinaceae which is widely distributed in Southeast Asia, Australia and Pacific islands. Among the four genera of the family Casuarinaceae, species of *Casuarina* are cultivated widely in the tropical and subtropical regions of the world. Currently *C. equisetifolia, C. junghuhniana, C. cunninghamiana, C. cristata, C.obesa and C. glauca* are the most successfully introduced, cultivated and subjected to tree improvement program. The selection of a species depends on adaptability to various ecological environments, drought tolerance and saline-alkali tolerance and having early rapid growth.

In India, *C. equisetifolia* was introduced during the 19th century and is now estimated to be under cultivation in around half a million hectares mainly in the Peninsular region. Apart from fuel, the wood is extensively used for papermaking and of late is a preferred choice for biomass-based power generation. The straight cylindrical stems find use in rural house building and as scaffolds in construction sites. It is the principal species for developing shelterbelts in coastal areas and windbreaks for protecting agricultural crops. Clonal plantations of this species are expanding in India, China, Vietnam and Egypt that are raised from the outstanding phenotypes selected from newly introduced provenances.[3,18,29]

The advent of molecular techniques has enhanced population genetics study which plays a key role in detecting genetic variation or polymorphism. Techniques have become indispensable markers of analysis and are indicative of neutral genetic variation. Molecular data use characters that evolve typically at higher rates but with a great diversity represented at different loci within the genome.[24] The choice of molecular marker technique depends on the mode of inheritance, reproducibility and simplicity. A wide range of molecular markers have been developed for tropical and conifers species. Molecular markers such as RAPD, ISSR, SSR, AFLP and RFLP have been used in cultivar fingerprinting, seed purity test and germplasm identification for plant species.[8,17]

Among the various molecular markers currently available, simple sequence repeats (SSR), also known as microsatellites are widely accepted as highly reliable markers. SSRs have proven to be abundant and well distributed throughout the plant genome. They are co-dominant, detect high levels of allelic diversity and are assayed efficiently by the PCR.[15] SSR markers have become ideal for genetic map construction[19], identification of clone[7], identification of species and hybrids, determination of paternity[27] and marker- assisted selection.[28] SSRs are generally categorized into two common groups based on

their origins, genomic SSRs from genomic sequences and expressed sequence tag (EST)-SSRs fromtranscribed RNA sequences. As one of the powerful next-generation sequencing (NGS) methods, Illumina RNA-seq has proven to be robust and efficient with higher throughput and much lower cost[5,31] than other systems and has been used in many plants to reveal gene annotation and expression under biotic and abiotic stresses.[22]

The genetic diversity of *Casuarina* species from natural and introduced populations has been studied with various types of DNA markers including RAPDs, ISSRs[10,32]. SSRs[30,33] and SCARs[16]. *C.equisetifolia* genome has not yet been fully sequenced. Fourty two SSR markers are from 86,415 ESTs of *C. equisetifolia* and *C. junghuhniana*[11]. However, the number of SSR markers reported for Casuarina species is still far fewer than other commonly planted tropical tree species such as *Populus* species[12], *E. camaldulensis*[4], *E. globulus*[1,34] and *E. Urophylla.*[2] SSRs are suggested for identification of species and hybrids in species such as Quercus,[25] Pinus[6], Juglans[21] and Eucalyptus[4].

These markers were used for identification and differentiation of individuals, species and provenances and to explore within and between population variations. It is necessary to develop more novel cross-species transferable SSR markers for promoting. The present study was conducted with the following objective to study the genetic diversity between four *Casuarina* species (*C. cunninghamiana*, *C. equisetifolia*, *C. cristata* and *C. junghuhniana*) and the hybrids between *C. equisetifolia* and *C. junghuhniana* using EST-SSR marker.

Material and Methods

The details on the *Casuarina* accessions used in this study are presented in table 1. Five individuals from each species, five hybrid clones of *C. equisetifolia* × *C. junghuhniana* and six open-pollinated progenies of *C. cunninghamiana* and *C. cristata* (3 individuals each) were used for analyzing EST-SSR polymorphism. The hybrid accessions of *C. equisetifolia* × *C. junghuhniana* were selected from a progeny trial established with fullsib families produced through control pollination.

The progeny accessions of *C. cunninghamiana* and *C. cristata* were obtained from open-pollinated seeds collected from selected trees in the species trial to determine whether any cross-pollination occurred across the four species planted in the trial. Actively growing tips of the cladodes from the identified accessions were collected and stored in the freezer at -80 ^{o}C for the extraction of genomic DNA.

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. Genomic DNA was isolated and purified using QiagenDNeasy Plant mini kit (Qiagen, USA). The purity, size and integrity of DNA were determined by using PicodropSpectrophometer (Picodrop microliter spectrophotometer version 3.01, UK). The SSR loci targeting di- and tri-nucleotide repeats were obtained from the GenBank EST sequences of *Casuarina glauca*. The EST-SSRs were identified using WebSat [14] and primers for SSR amplification were designed by Primer3 software [23] integrated into WebSat.

The details of the primers sequences, NCBI accession ID, target SSR motif, melting temperature and expected product size are given in table 2. SSR-PCR amplifications were carried out in the reaction mixtures of 10µl containing 1x PCR buffer (with 200ng BSA), 0.4mM dNTPs, 0.4µM each of forward and reserve primes, 1.0 unit of Taq DNA polymerase (MBI Fermentas) and 10-20ng of template DNA. All the PCR reactions were carried out in BIO-RAD Thermal Cycler using the following reaction conditions: 94^{o}C for 5 min-initial denaturation and 30 cycles of 94^{0}C for 1 min- denaturation, 56 ^{o}C– 61 ^{o}C for 30s – anneaing, 72^{o}C for 2 min- extension followed by final extension of 72 ^{0}C for 7 min.

PCR products were size-separated using 5% denaturing polyacrylamide gels of size 21cm x 50cm (Sequi-Gen System, BIO-Rad, USA) containing 7 M urea and 1 X TBE buffer. After separation, the gels were subjected to silver staining as described by Zhang et al[35].

The microsatellite data generated were scored (bp) manually using the 50bp size standard (MBI Fermentas, USA) after required image adjustments in Adobe Photoshop^{TM}1 and were recorded for further statistical analysis. CERVUS version 3.0.3[9] was used to determine the number of alleles (N_A) per locus, polymorphic information content (PIC) observed (H_O) and expected heterozygosity (H_E). Darwin v.5 software[20] was used to to estimate the genetic dissimilarity among the casuarina accessions with simple matching coefficient and dendrogram was constructed with unweighted pair group method with arithmetic averages (UPGMA) after 100 bootstrap replications.

Results and Discussion

Thirty one individuals from four different species of *Casuarina* (*C. cristata, C. cunninghamiana, C. equisetifolia* and *C. junghuhniana*) and the hybrids generated between *C. equisetifolia* and *C. junghuhniana* were amplified using four polymorphic EST-SSR loci. Out of 10 EST-SSR loci analyzed, four were selected based on the clear banding pattern and no stutters (Fig. 1a and b). Other loci were amplifying the genomic DNA of all the species, however, need further optimization.

The number of alleles (N_A), the PCR product size, polymorphic information content (PIC) and the heterozygosity of each of these four loci are shown in table 3. The N_A ranged from 11 to 20 and the H_O and H_E ranged from 0.38 to 0.54 and 0.78 to 0.94 respectively.

Table 1
Details on accessions of *Casuarina* species and their inter-specific hybrids used in this study

Accession Number	Accession Name	Prov. & Tree ID	Provenance	Location
1	CCU1	16	9km N Rollingstone, Australia CSIRO No.13519	Karunya
2	CCU2	16		
3	CCU3	16		
4	CCU4	16		
5	CCU5	16		
6	CCR1	18	Dalby Moonie Hwy, QLD, Australia CSIRO No.17757	Karunya
7	CCR2	18		
8	CCR3	18		
9	CCR4	18		
10	CCR5	18		
11	CJ1	55	Second generation progeny trial	Salem
12	CJ2	88		
13	CJ3	23S		
14	CJ4	23K		
15	CJ5	86		
16	CE1	164	Second generation progeny trial	Karur
17	CE2	113		
18	CE3	166		
19	CE4	175		
20	CE5	183		
21	CH7	7	Inter-specific Hybrids of CE and CJ	IFGTB Clone Bank
22	CH1	17		
23	CH2	18		
24	CH3	22		
25	CH4	25		
26	CCR P1	119 (17/9)	Inglewood, QLD, Australia CSIRO No.15239	Karunya
27	CCR P2	121(18/2)	Dalby Moonie Hwy, QLD, Australia	
28	CCR P3	122(18/11)		
29	CCU P1	110(16/1)	9km N Rollingstone, Australia	
30	CCU P2	111(16/10)		
31	CCU P3	118(16/14)		

Note: CCU- *C. cunninghamiana*, CCR- *C. cristata*, CE- *C. equisetifolia*, CJ- *C. junghuhniana*, CH- Inter-specific hybrids of CE and CJ, CCUP- *C. cunninghamiana* progeny and CCRP- *C. cristata* progeny

Table 2
Characteristics of EST-SSR primers used for analyzing *Casuarina* species and hybrids

NCBI Accession Number	SSR code	Forward sequence 5' -3'	Reverse sequence 5' -3'	SSR motif	Tm (°C)	Expected product size (bp)
FQ373954	SSR1	GTCAAGGTCTTTCCTTTAGCCA	GTTCCTTAAATTGGTGGTAGGC	$(TCT)_{10}$	58.9	332
FQ374771	SSR2	TGTCCTACCGTTTCAGTTTGTG	GTCTTCTTCGTCAGCATCTCCT	$(GAC)_{12}$	60	234
FQ376734	SSR3	AGCTCTGCTTCACTTGCTCTCT	CAGCACATACGCCTAGTTTGAG	$(GTG)_6$	60	193
FQ373403	SSR8	TCAGGAACAACATATCGCGTAG	TTAATATCCGACACTGCACCTG	$(AG)_{25}$	60	360
FQ367805	SSR10	GGGGACAGACAAGCACAAAC	CCTTGCACGTACTCCTGGTATT	$(AG)_{17}$	60	208

Mean number of alleles per locus was 15.0 and the polymorphic information content was 0.85. The present study confirms the findings of Kullan et al[11] reported that EST-SSR markers revealed high level of polymorphism, detecting a total of 829 alleles with an average of 17 alleles per locus. Polymorphic information content (PIC) values ranged from 0.32 to 0.93 with an average of 0.78 per locus.

The average observed (Ho) and expected heterozygosity (He) obtained were high and fairly similar in *C. cunninghamiana* and *C. junghuhniana*, thereby suggesting highly heterogeneous nature of Casuarina. In recent studies Xiuyu et al[30], reported the number of alleles per locus (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e) and polymorphic information content (*PIC*) averaging at 5.5, 0.72, 0.86 and 0.63 respectively in *C. equisetifolia*. The rates of cross-species transferability ranged from 96.9% (*C. glauca* Sieber ex Sprengel) through 97.8% (*C. cunninghamiana* Miquel) to 99.1% (*C. junghuhniana* Miquel). Yasodha et al[33,34] confirmed the transferability of Eucalyptus SSR markers across two species of *C. equisetifolia* and C. glauca. Among 42 Casuarina SSR markers, 80% were transferable across four species of *C. cunninghamiana*, *C. junghuhniana*, *C. equisetifolia* and *C. glauca*.[11]

The dendrogram of the EST-SSR data was able to clearly distinguish the pure species and hybrid individuals and the uniqueness of each individual was also established. Four major clusters were produced on the basis of species and their hybrids (Fig. 2). Two distinct clusters separated out *C. cristata* and *C.cunninghamiana* (provenances from Australia) from other species and their hybrids. The six open-pollinated progeny accessions of *C. cunninghamiana* and *C. cristata* collected from the species-provenance trial showed no evidence of hybridization across species. But within each of these species, trees directly sampled from the species-provenance trial and the progeny raised from seeds collected from them formed distinct sub-clusters.

Another major cluster consisted of individuals of *C. equisetifolia* and *C. junghuhniana* (provenances from Asian countries) and the fourth cluster included the hybrid individuals. Interestingly, the cluster with hybrids merged with their parent species confirming their hybridity status (Fig. 2).

Table 3
Number of alleles (N_A), PCR product size, polymorphic information content (PIC) and heterozygosity content of different SSR loci used for *Casuarina* species

SSR code	Product size (bp)	No of alleles produced	H_O	H_E	PIC
SSR1	332	19	0.387	0.786	0.744
SSR2	234	17	0.484	0.907	0.883
SSR3	360	20	0.387	0.948	0.928
SSR4	233	11	0.548	0.875	0.846
Mean	289.75	15	0.451	0.879	0.85

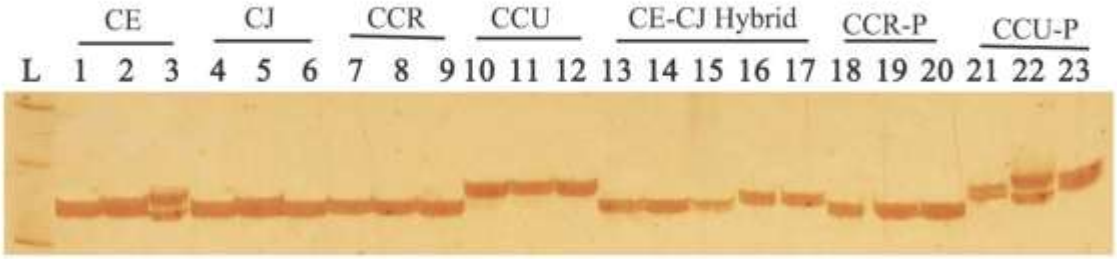

Fig. 1a: SSR primer (SSR1) profiles for four species of *Casuarina* and its Hybrid

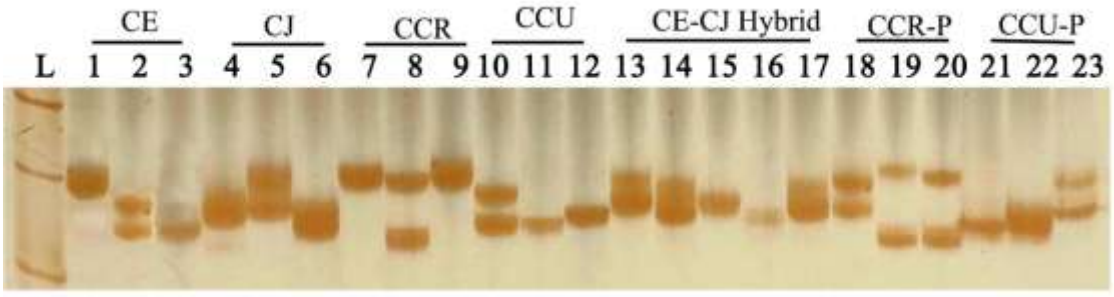

Fig. 1b: SSR primer (SSR3) profiles for four species of *Casuarina* and its Hybrid

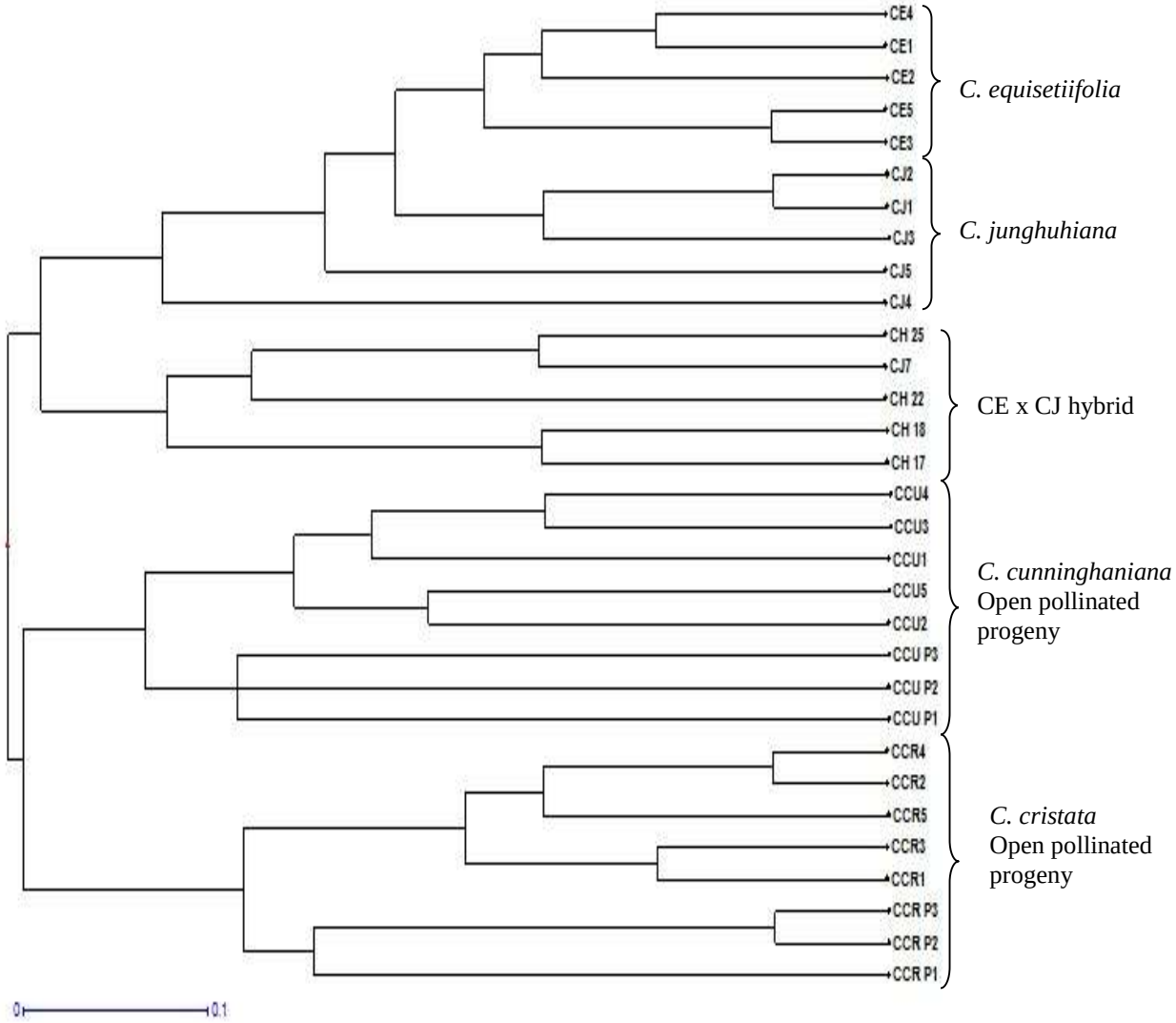

Fig. 2: Dendrogram showing genetic relationship among 31 *Casuarina* accessions based on UPMGA analysis using 4 SSR primers

Encouraged by the high productivity obtained from clonal plantations of Eucalyptus, there is a heavy demand for clones of *Casuarina* that can increase wood production in the short-term. During the last decade, the extent of clonal plantations of *Casuarina*has steadily increased which calls for revising the strategy for deployment of genetically improved planting material in commercial plantations. Seeds have been the major form of deploying the breeding output in plantations considering the high density plantation and the preference of smallholding farmers. The stocking in plantations has been reduced by adopting wide spacing and convinced by the increase in wood production, farmers now prefer clones over seedlings.

New clones have to be developed which can match with the diverse planting environments and end uses of *Casuarina* in India. Inter-specific hybrid clones provide the opportunity to tailor-make clones specifically for the given planting environment and desired end product/service with amenability to large scale commercial propagation.

The EST-SSR markers were shown to be highly efficient and polymorphic and found to be useful for distinguishing the closely related inter-specific hybrid genotypes. These EST-SSR markers would therefore facilitate new avenues for genetic improvement program in *Casuarina* species and related taxa.

These molecular markers will be widely used in the advanced generation breeding programmes of *Casuarina* to increase selection and breeding efficiency for meeting the challenges mentioned above. The unique breeding system of *Casuarina* needs an efficient marker system to effectively manage the breeding populations and to validate the selections made for meeting different needs. The present study has clearly shown the ability of EST-SSR markers in

discriminating the species, individuals within species and hybrid combinations of different species even with a low number of loci.

Some of the loci showed highly species-specific profiles (Fig. 1a and b) which will be useful in quantifying introgression across species. Since Casuarinas are wind-pollinated, breeding populations and seed orchards are likely to face contamination with pollen sources from same or different species. DNA marker profiles will increase efficiency of selecting candidates for breeding and propagation and will also improve the precision of ranking of trees for thinning. Inter-specific hybrids can be validated at an early stage so that field testing can be restricted to only the hybrid individuals and they can also be traced in the large scale commercial production.

Conclusion

The preliminary results obtained in the present study indicate the potential of identifying trait-specific EST-SSR markers in future. All the four species investigated in the present study have a wide distribution range covering diverse climatic and soil conditions.

As a result, they possess a large amount of genetic variation in terms of adaptability, growth and wood traits. Since the predominantly dioecious sexual system and unisexual flowers in *Casuarina* offer scope for production of large number of crosses through control pollination, inheritance of markers linked to traits of economic importance and adaptability characteristics in a large mapping population can be accomplished relatively easily compared to insect-pollinated species with bisexual flowers.

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