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Genetic Diversity Analysis in *Casuarina* and *Allocasuarina* Species Using ISSR Markers

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One hundred twenty individual samples belonging to three *Casuarina* (*C. junghuhniana C. glauca* and *C. equisetifolia*) and two *Allocasuarina* species (*A. huegeliana* and *A. littoralis*) grown in a species trial garden at Panampally Research Station, Kerala, India were studied for genetic diversity estimation. Seven ISSR primers targeting 2 or 3 nucleotide repeats anchored at 3' or 5' end were used and 241 polymorphic amplicons were scored. The size of the amplification products ranged from 220-1710 bp. Among the genera studied, percent polymorphism were 40.6% and 23.1% in *Casuarina* and *Allocasuarina* respectively. At species level, the highest percent polymorphic loci was observed in *C. equisetifolia* (18.6%) while the lowest was registered in *A. littoralis* (4.8%). Analysis of molecular variance (AMOVA) showed that a large proportion of genetic variation (92.36%) resided among species, while only 8.64 per cent resided among individuals within species. The estimation of genetic diversity will be an important tool in *Casuarina* hybridization program.

Key words: Allocasuarina, Casuarina, Inter Simple Sequence Repeats, genetic diversity

Casuarina is a multipurpose tree, widely grown in tropical and subtropical countries for fuel wood, pulp, charcoal, fencing material, buffer zones at beaches and poles Djogo (1996). They are also planted for reforestation, shelterbelt, firebreaks, shade, ornamental, soil fertility management and rehabilitation of lands (Midgley *et al.* 1981; Djogo, 1996) and are excellent soil reformers and increase soil fertility through nitrogen fixation (Dommergues *et al.* 1990). Currently casuarinas are grouped under four genera, with over 90 species (Wilson and Johnson, 1989; Moneur *et al.* 1997). Among the four genera, the species of *Casuarina* and *Allocasuarina* are commercially cultivated in many tropical and subtropical regions of the world while the other two genera, *Ceuthostoma* and *Gymnostoma* occur as wild species only. *Casuarina* comprises of 17 species distributed throughout Southeast Asia and Australia (Pinyopusarerk and House, 1993) while *Allocasuarina* comprising of 59 species are endemic to Australia (Wilson and Johnson, 1989). Taxonomic and phylogenetic relationships within the species of casuarinas have been studied using chromosome number, pattern of geographical distribution (Barlow, 1959; 1983), diversification in the morphological characteristics (Wilson and Johnson, 1989; Kumar and Gurumurthi, 2000), DNA markers (Yasodha *et al.* 2004; Ho *et al.* 2002; 2004), *matK* and *rbcL* gene sequences (Sogo *et al.* 2001) to distinguish the members of *Allocasuarina*, *Casuarina*, hybrids of *Casuarina* and *Casuarina equisetifolia* clones.

Selection and breeding in applied tree improvement programs exploit variation at the individual level to locate and combine the existing natural variation into improved genotypes. Traditionally, morphological observations and progeny tests have been used as descriptors of genetic diversity, but they failed to reveal their exact taxonomic affinity since most of the morphological characters are subject to phenotypic plasticity and are influenced by environmental factors.

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Molecular markers have proved to be a valuable tool in the characterization and evaluation of genetic diversity within and between species and populations (Hou *et al.* 2005). Inter-simple sequence repeat PCR (ISSR–PCR) is a simple, cost-efficient, robust, reproducible, multi-locus marker method which is extremely useful in determining genetic variability both at inter and intra-specific level (Joshi *et al.* 2000; Reddy *et al.* 2002). ISSR has been extensively used for several applications in molecular taxonomy (Wolfe *et al.* 1998; Blair *et al.* 1999; Ruas *et al.* 2003) and genetic diversity analysis in species like tea (Bahulikar et al., 2004; Wai et al., 2005), *Casuarina* (Yasodha *et al.* 2004) and *Eucalyptus* (Gemas *et al.* 2004; Balasaravanan *et al.* 2005) Teak (Narayanan *et al.* 2007), and *Gemliana* (Singh *et al.* 2008) .

The present study characterizes the genetic diversity in three *Casuarina* (*C*. *equisetifolia*, *C*. *junghuhniana* and *C*. *glauca*) and two *Allocasuarina* (*A*. *huegeliana* and *A*. *littoralis*) species for use in casuarina breeding program.

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 center, 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.

DNA extraction and amplification conditions

Total genomic DNA was extracted from 100mg of fresh juvenile needles collected from *C. glauca* and *C. equisetifolia* (25 individuals), *C. junghuhniana* (24 individuals), *A. huegeliana* and A*. littoralis* (23 individuals) by using Qiagen DNeasy plant mini kit (Qiagen, Hilden, Germany) as described by the manufacturer. Extracted DNA was quantified using a spectrophotometer and by comparing band intensities with known standards of lambda DNA (Bangalore Genei Ltd, India) on 0.8% agarose gels. ISSR-PCR amplifications were performed in a 10µl reaction volume containing about 30ng of template DNA, 1.0µl 10X PCR buffer (Bangalore Genei Ltd, India), 2.5mM $MgCl_2$, 0.4mM dNTPs, 100nM primer (synthesized at Sigma-Aldrich, USA) and 0.3U *Taq* DNA polymerase (Bangalore Genei Ltd, India). A total of seven primers were used in the study and the details are provided in (Table 2). PCR amplifications were carried out in a programmable thermal cycler (PTC-200, MJ Research, Inc., USA) with following conditions: Initial denaturation (3 min, 94 °C), followed by 35 cycles consisting of denaturation (30 sec, 94 °C), annealing (30 sec, 50 °C), extension (1 min, 72 °C) and a final extension (10 min, 72 °C). Amplification products were resolved in a 2 per cent agarose gel with 1X TAE buffer at 70V for two hours 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.

Data analysis

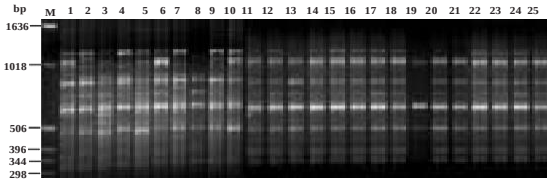
ISSR amplicons were scored as presence (1) or absence (0) in each individual and a similarity matrix was derived from the binary data using Nei and Li's similarity indices (Nei and Li, 1979). The similarity matrix was used in a UPGMA cluster analysis and a principal coordinate analysis by employing the software NTSYS-pc (Rohlf, 1992). The percentage of polymorphic loci (Kimura and Crow, 1964), gene diversity (Nei, 1973), Shannon's information index (Lewontin, 1972) and genetic identity/ distance (Nei, 1972) was calculated using POPGENE, v. 1.31 (Yeh *et al.* 1999). Tree confidence was also tested by a bootstrap analysis (Yap, 1996) with 1000 replications. The genetic structure of population was analyzed by Analysis of molecular variance (AMOVA) using the Arlequin, v. 2.0 software described by (Excoffier *et al.* 1992). The populations were divided in to two groups comprising of *Casuarina*

and *Allocasuarina* species and the number of permutations for significance testing was set as 1023 for all analysis.

Results and Discussion

The amplification details obtained with the seven ISSR primers for the two *Allocasuarina* and three *Casuarina* species are given in Table 2. A total number of 241 scorable PCR products were detected in the size range of 220 to 1710 bp. The mean number of bands produced per species was 48.8. Total number of amplified loci per primer for all the five species varied from 26 (with $(GA)_8$ R) to 44 (with TA$(CAG)_4$). The maximum number of polymorphic bands and per cent polymorphism were 21 and 51.2 respectively, when amplified with primer UBC810. An ISSR profile generated by primer UBC 842 in *C. equisetifolia* is shown in figure 1.

Figure 1.ISSR profile of *Casuarina equisetifolia*, amplified with primer UBC 842



Lanes 1 to 25: *C. equisetifolia* individuals; Lane M: Marker (1 Kb ladder, Gibco BRL, Ltd, USA)

Among the five species, high per cent polymorphic loci was observed in *C. equisetifolia* (18.55), whereas, the minimum was in *A. littoralis* (4.84). Maximum mean gene diversity and Shannon's information index was recorded in *C. equisetifolia* with values of 0.0587 and 0.0878 respectively and the minimum was in *A. littoralis* with 0.0176 and 0.0261 respectively (Table 3). The dendrogram showed two main clusters, one with *C. equisetifolia*, *C. glauca* and *A. littoralis* and another with *C. junghuhniana* and *A. huegeliana* (Figure 2). The combined principal coordinate (PCO) segregated each species except *C. equisetifolia* and C*. glauca* (Figure 3).

Nei's genetic identities and distances between the three *Casuarina* and two *Allocasuarina* species are showed in Table 4.

Figure 2. Dendrogram showing similarity among five species obtained by ISSR markers using Jaccord coefficient. Values at nodes refer to bootstrap values

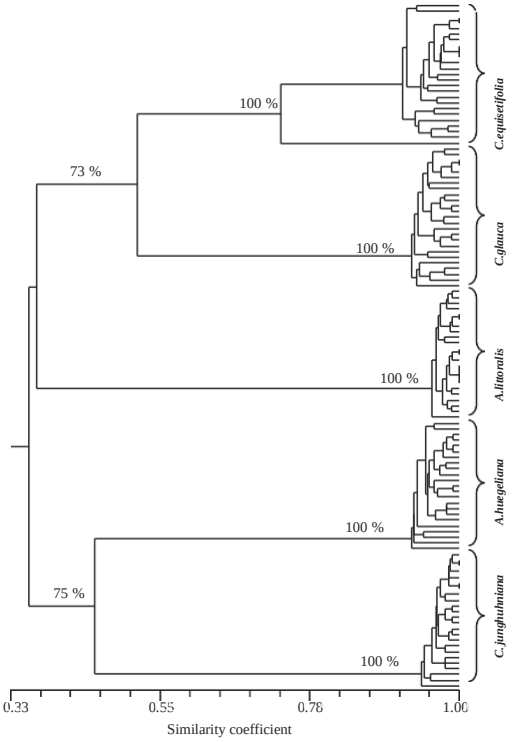
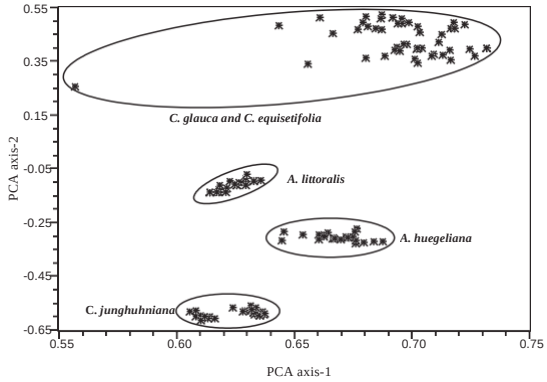


Figure 3. PCO scatter plots showing distribution of *Casuarina* and *Allocasuarina* species



The pair wise genetic identity among species ranged from 0.535 (*A. huegeliana* and *A. littoralis*) to 0.759 (C*. equisetifolia* and *C. glauca*) respectively. The genetic distance was high between *A. littoralis* and *A. huegeliana* (0.625) and low between *C. glauca* and *C. equisetifolia* (0.276). Analysis of Molecular Variance (AMOVA) estimated the percentage of variance

Table 1. Details of *Casuarina* and *Allocasuarina* species for ISSR analysis

Spices Name	Chromosome Number (2n)	CSIRO seedlot Number	No. of Parent trees	Locality	Altitude (m)	Latitude (N)	Longitude (E)
C. *equisetifolia ssp. equisetifolia*	18	19129	4	Lakei/sibur Bako MLAY	40	1° 44'	111° 29'
C. *gluca*	18	15941	9	Burrum Heads QLD	100	$25^0$12'	152° 37'
A. *huegeliana*	26	15801	4	Sanford Rock WA	380	31° 13'	118° 46'
A. *littoralis*	22	13876	5	Chili Cks QLD	80	12° 42'	143° 20'
C. *junghuhniana ssp. junghuhniana*	18	19489	10	Kapan Kupang INDO	700	10° 13'	123° 38'

MLAY: Malaysia; QLD: Queensland; WA: Western Australia; INDO: Indonesia

among genera, among species within genera and within species. Among genera, variation components value was negative (-0.47; p>0.26393). This explained the absence of a genetic structure between the two genera for the targeted loci. In out crossing species, loci from different populations can be more related to each other than loci from the same population. A high genetic variation of 92.36 per cent resided among species within genera, while only 8.64 per cent resided among individuals within species (Table 5).

Table 2. List of ISSR primers used for the genetic analysis of *Casuarina* and *Allocasuarina* species showing number and size range of amplified bands for each primer

Primer code	Nucleotide sequence 5' to 3'	Amplification range (bp)	No.of.bands scored	PB	Per cent polymorphism
5' anchored					
$R(CA)_7$	GRTRCYGRTRCACACACACACACA	270-1636	34	11	32.4
$T(GT)_9$	CRTAYGTGTGTGTGTGTGTGTGT	365-1018	36	10	27.8
$TA(CAG)_4$	ARRTYCAGCAGCAGCAG	220-1710	44	13	29.5
$RA(GCT)_6$	AYARAGCTGCTGCTGCTGCTGCT	220- 700	28	5	17.9
3' anchored					
$(GA)_8R$	GAGAGAGAGAGAGAGARGY	320- 1035	26	12	46.2
UBC810	GAGAGAGAGAGAGAGAT	335-1225	41	21	51.2
UBC842	GAGAGAGAGAGAGAGAYG	330-1140	32	7	21.9
		Mean	34.4	11.3	32.4
		SD	6.5	5.1	12.2

Note. PB- No of polymorphic bands, SD – Standard Deviation.

The Knowledge on genetic variation is the first step in designing a genetic improvement program and for conservation. Genetic diversity is essential for the long-term survival of tree species to avoid risk of extinction. The loss of genetic variation decreases both the short-term and the long-term

adaptability of populations in variable and changing environments (Hamrick, 1994; Young *et al.* 1996). Molecular marker techniques such as RAPD, ISSR and SSR have been used to assess the genetic diversity and phylogeny of *Casuarina* species (Ho *et al.* 2002; Yasodha *et al.* 2004; 2005). The genetic structure of *Casuarina* species found in this study was characterized by low genetic variation within populations (8.11%) and high genetic differentiation between populations (92.36%). Similar result was reported by (Li and Ge, 2001) in seven *Psammochloa villosa* populations using ISSR markers. Their AMOVA revealed that 87.46 per cent of the variance component was among populations and 12.54 per cent within populations. In this context, the low level of genetic variation within populations and high genetic differentiation among populations in *Casuarina* species may be attributed mainly to the seed dispersal and natural selection. High levels of genetic differentiation between populations were also detected based on Nei's genetic diversity analysis (63.8%) and a similar trend was documented in *Glyptostrobus pensilis* populations using ISSR primers (Li and Xia, 2005).

Table 3. Genetic variability within *Casuarina* and *Allocasuarina* species detected by ISSR analysis

Species	na	ne	h	I	P(%)	N
C.*equisetifolia*	1.1855(0.390)	1.1024(0.266)	0.0587(0.144)	0.0878(0.208)	18.55	25
C.*glauca*	1.1371(0.345)	1.092(0.260)	0.0515(0.139)	0.0757(0.200)	13.71	25
A.*huegeliana*	1.1452(0.354)	1.0812(0.229)	0.0483(0.129)	0.073(0.189)	14.52	23
C.*junghugniana*	1.0968(0.297)	1.0757(0.247)	0.0407(0.131)	0.0582(0.185)	9.68	24
A.*littoralis*	1.0484(0.215)	1.0302(0.148)	0.0176(0.083)	0.0261(0.121)	4.84	23

Note. Sample sizes (N), Observed number of alleles per locus (na), The effective number of alleles per locus (ne), Gene diversity (h), Shannon's Information index (I), percentage of polymorphic loci (P).

In *C. equisetifolia*, Ho *et al.* (2002) documented average mean gene diversity 0.147 and 0.140 in international provenances and hybridization orchard respectively using RAPD primers. In our study, *C. equisetifolia* documented comparatively low level of mean gene diversity (0.0587). This may be due to the less number of parent trees (4 numbers) used for bulking of seeds and the limited population size used in the present study. Our results show that the genetic diversity in *A. littoralis* is lower than that of those congeners at the species level and at the population level. This might have resulted from its restricted geographical distribution and isolated small populations. The genetic relationship of *C. equisetifolia* and *C. glauca* revealed in the present study is in accordance with observation made earlier Ho *et al.* (2002) and Yasodha *et al.* (2004).

Table 4. Genetic identity and genetic distance among the species of *Casuarina* and *Allocasurina*

Species		C.eq	C.gl	A.hu	C.ju	A.li
C. equisetifoloia	(C.eq)	***	0.759	0.667	0.635	0.632
C. glauca	(C.gl)	0.276	***	0.568	0.536	0.579
A. huegeliana	(A.hu)	0.404	0.565	***	0.612	0.535
C. junghuhniana	(C.ju)	0.455	0.623	0.491	***	0.56
A. littoralis	(A.li)	0.459	0.547	0.625	0.58	***

Genetic identity (above diagonal) and genetic distance (Below diagonal)

Earlier studies showed the phenetic relationship between species from four genera within family Casuarinaceae using *matK* and *rbcL* gene sequence data revealing the distinctness of the two genera *Allocasuarina* and *Casuarina* (Sogo *et al.* 2001; Steane *et al.* 2003). Yasodha *et al.* (2004) also reported separate grouping of the two genera using ISSR markers. The present study, however did not show the separate group of *Casuarina* and *Allocasuarina* because C*. junghuhniana* being a species natively distributed only in Indonesia was not used in earlier studies (Table 1). This study presents a detailed analysis of genetic relationship between five species of casuarinas which can determine the suitability of these species in breeding programs.

Table 5. Analysis of molecular variance (AMOVA) for five species using ISSR primers

Source of variation	d.f	SSD	MSD	Variance component	% Total	P-value*
Among genera	1	584.478	584.478	-0.127	-0.47	> 0.2639
Among species within genera	3	1809.82	603.275	24.932	92.36	< 0.001
Within species	115	251.832	2.190	2.189	8.11	< 0.001

Note. Degrees of freedom (d.f), Sum of squares (SSD), (MSD) Mean sum of squares, The percentage of the total variance (% Total). * Significance tests after 1023 random permutations

Conclusion

In tree improvement programs, selection of parents are traditionally done through morphological observations and progeny tests are conducted to assess the genetic diversity. With the advent of molecular marker techniques, diversity estimations using DNA markers have become important in designing genetic improvement programs. The present study reveals the genetic relationship with in and between three species of *Casuarina* and two species of *Allocasuarina* belonging the family Casuarinaceae using ISSR-PCR. Among the genera studied, percent polymorphism were 40.6 per cent and 23.1 per cent in *Casuarina* and *Allocasuarina* respectively. At species level, the highest percent polymorphic loci were observed in *C. equisetifolia* (18.6%) while the lowest was registered in *A. littoralis* (4.8%). Partitioning of genetic diversity showed that 92.36 per cent genetic variation resided among species, while only 8.64 per cent resided among individuals within species. The diversity estimates generated from the present study will help in selecting populations for *Casuarina* breeding program.

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