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Morphological and Molecular Diversity among *Casuarina* and *Allocasuarina* Species

R. Kamalakannan, Santan Barthwal, P. Chezhian, T. Balasaravanan,
R. Yasodha, K. Gurumurthi and Modhumita Ghosh
Plant Biotechnology Division, Institute of Forest Genetics and Tree Breeding,
Forest Campus, R.S. Puram, Coimbatore, Tamil Nadu, 641 002, India

Abstract: Morphological and genetic diversity among three species of *Casuarina* (*C. equisetifolia*, *C. glauca* and *C. junghuhniana*) and two species of *Allocasuarina* (*A. huegeliana* and *A. littoralis*) were studied with eighteen morphometric parameters and seven ISSR primers. The morphometric parameters that differentiated most species included plant height, branch length, internode length and teeth length and revealed a significant diversity among the species studied. ISSR-PCR analysis was also conducted to estimate the genetic relationship among and within the species. Seven ISSR primers were amplified in 50 individuals belonging to the five species. A total of 66 polymorphic bands amplified with the amplicon size ranging from 220 bp to 1140 bp. The dendrogram generated from ISSR and morphometric markers clustered *C. equisetifolia*, *C. glauca* and *A. littoralis* in to one group while *C. junghuhniana* and *A. huegeliana* grouped separately. The study revealed that the diversity estimation by conventional method using morphometric parameters and advanced technique utilizing molecular data can be used synergistically in diversity estimation studies for population/parent selections in breeding programs of tree species.

Key words: Casuarinaceae, *Casuarina*, *Allocasuarina*, genetic diversity, ISSR, morphometry

INTRODUCTION

The family Casuarinaceae belongs to the Gondwanic family and consist of four genera (*Allocasuarina*, *Casuarina*, *Ceuthostoma*, *Gymnostoma*) and 97 species including monoecious or dioecious shrubs and trees (Chen, 1982; Johnson and Wilson, 1989). 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* 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 (Yasodha *et al*., 2004). Casuarinas are cultivated in China, India, South East Asia, Malaysia and Pacific islands and used for landscaping, pulp, lumber, medicine, tannin, dye and sand-shifting control in coastal areas (Pan *et al*., 1996). About 15 species of *Casuarina* and *Allocasuarina* have been recognized for multiple utilization (Doran and Hall, 1983) and were first introduced in India as a fuel wood species during the late 19th century (Nicodemus *et al*., 2001). Broad difference in growth and morphological traits have been reported from different regions. Steane *et al*. (2003) reported the extreme morphological reduction in this family, as well as the unique combination of morphological traits like drooping equisetoid twigs, reduced scale-like leaves in whorls forming toothed sheaths at each node, inflorescences with alternating whorls of tooth-like bracts and reduced flowers, wind pollination, wood 'cone'-like infructenscences and winged samaras as fruits. Based on the earlier classification, the genera *Allocasuarina* was grouped with *Casuarina* due to its morphological relatedness but subsequently both genera was separated due to distinguishing characters like mature samara color, thickness of cone bracteoles and teeth per whorl (George, 1989).

Selection and breeding in tree improvement programs utilize variation at the individual level to locate and repack

Corresponding Author: Modhumita Ghosh, Plant Biotechnology Division, Institute of Forest Genetics and Tree Breeding, Forest Campus, R.S. Puram, Coimbatore, Tamil Nadu, 641 002, India
Tel: +91 0422 2431540 Fax: +91 0422 2430549

the existing natural variation into improved genotypes. International *Casuarina* workshops held in Cairo (Zhong, 1990) and Vietnam (Pinyopusarerk *et al*., 1996) strongly recommended a tree improvement program in *C. equisetifolia*. The selection criteria were identified as crown shape, branch angle, length of branchlet, cone production, competence to root form the lower branches, dbh, height, volume, natural pruning, nodule weight and disease incidence were considered for clonal propagation and selection of superior performing *C. equisetifolia* trees (Kondas, 1983; Kondas *et al*., 1985; Paramathma *et al*., 1994; Kumar, 1995). Recently reproductive traits and physiological traits like photosynthesis, stomatal conductance and transpiration and intercellular carbon di oxide concentration were recommended for selection of high yielder (Nicodemus *et al*., 2001; Balasubramanian and Gurumurthi, 2001).

In the present study, few of the morphometric traits were evaluated for their utility in generating diversity estimates in *C. equisetifolia* and its related species like *C. glauca* and *C. junghuhniana* and its species from sister taxa like *A. huegeliana* and *A. littoralis*. The data was compared with molecular estimates generated using ISSR markers.

MATERIALS AND METHODS

Plant materials: Seeds of three *Casuarina* species (*C. junghuhniana* Miq., *C. glauca* Sieb Ex Spreng and *C. equisetifolia* L) and two *Allocasuarina* species (*A. huegeliana* (Miq.) L. Johnson and *A. littoralis* (Salisb.) L. Johnson) were obtained from 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, Kerala, India. Three years old plants were utilized for morphological studies and young needles were collected for DNA extraction. The morphometric parameters were recorded at the trial site and the molecular study was carried out in the Institute of Forest Genetics and Tree Breeding, Coimbatore.

Morphological characterization: Eighteen morphological (seven qualitative and eleven quantitative) characters were assessed in ten individuals randomly selected from each species trial (Table 3). Morphological characters like plant height, collar diameter, girth at breast height (GBH) and stem form considered for the study are economically important since the wood is used as poles for scaffolding, firewood and as pulp wood while the remaining characters like self pruning ability, disease resistance and needle characters have physiological relevance. Straightness of stem, pyramidal crown, upright branch angle, self-pruning, smooth bark surface and absence of disease incidence were taken as positive qualitative traits (Pinyopusarerk *et al*., 2004). For each quantitative character, the mean value of all the individuals were calculated and fixed at a standard point. Lower values than the standard point in number of permanent branches, branch length, needle length, needle thickness, number of nodes, number of teeth, teeth length and internodal length (physiologically important characters) were considered as positive traits while higher values were considered as negative traits. Higher values than the standard point in plant height, collar diameter and girth at breast height (economically important characters) were considered as positive traits, while lower values were considered as negative traits. Presence of positive trait was scored as "1" and the negative trait as "0".

ISSR analysis: DNA was isolated from 100 mg of juvenile needles collected from 10 randomly selected individuals of each species using Qiagen DNeasy plant mini kit (Qiagen, Hilden, Germany). Extracted DNA was quantified using spectrophotometer and by comparing band intensities with known standards of lambda DNA (Bangalore Genei, Ltd. India) on 0.8% agarose gel. A working solution of DNA (30 ng μL^{-1}) was prepared in sterile double distilled water. The PCR mixture (10 μL reaction) contained 1.0 μL, 10 × PCR buffer, 2.5 mM $MgCl_2$, 40 μM dNTPs, 0.3U Taq DNA polymerase (Bangalore Genei, Ltd., India), 100 nM ISSR primer (University of British Colombia and Centre for DNA fingerprinting and Diagnostics, Hyderabad, India) and

Table 1: Details of seed sources of *Casuarina* and *Allocasuarina* species used for morphological and molecular study

S.No	Species	Gametic No. (n)	CSIRO seed lot No.	Origin
1	*Allocasuarina huegeliana* (Miq.) L. Johnson	13	15801	Sanford Rock WA
2	*Allocasuarina littoralis* (Salisb.) L. Johnson	11	13876	Gordanand Chili Cks QLD
3	*Casuarina equisetifolia* L.	9	19129	Lakei/sibur Bako MLAY
4	*Casuarina glauca* Sieb Ex Spreng	9	15941	Burrum Heads QLD
5	*Casuarina junghuhniana* Miq.	9	19489	Kapan Kupang INDO

Table 2: List of ISSR primers used to study genetic relationship among the individuals of *Casuarina* and *Allocasuarina* species

Primer code	Nucleotide sequence
5' anchored	
R $(CA)_7$	5' GRTRCYGRTRCACACACACACACA 3'
T $(GT)_9$	5' CRTAYGTGTGTGTGTGTGTGTGT 3'
TA $(CAG)_4$	5' ARRTYCAGCAGCAGCAG 3'
RA $(GCT)_6$	5' AYARAGCTGCTGCTGCTGCTGCT 3'
3' anchored	
$(GA)_8$R	5' GAGAGAGAGAGAGAGARGY 3'
UBC810	5' GAGAGAGAGAGAGAGAT 3'
UBC842	5' GAGAGAGAGAGAGAGAYG 3'

15 ng of template DNA. Seven ISSR primers were used in the present study (Table 2) and the amplification reactions were carried out in Peltier Thermal Cycler (PTC-200, MJ Research, Inc.) using the following programme: 3 min at 94°C for initial denaturation; 35 cycles of 30 sec at 94°C for denaturation, 30 sec at 50°C for annealing and 1 min at 72°C for extension; 72°C for 10 min for final extension. The amplification products were resolved in a 2% agarose gel and stained with ethidium bromide.

Data analysis: The presence of a band was scored as "1" and absence as "0". The binary data scored from morphological observations and ISSR profiles were analyzed by statistical programmes. Using NTSYSpc (Rohlf, 1992), the similarity matrix was derived based on Nei and Li's similarity indices (Nei and Li, 1979). The similarity matrix was used for UPGMA cluster analysis and principal coordinate analysis. Analysis of polymorphism percentage, allele frequency (Kimura and Crow, 1964), gene diversity (Nei, 1973), Shannon's information index (Lewontin, 1972) and genetic identity/diversity (Nei, 1972) within and between the five species were estimated using POPGENE 1.32 software (Yeh *et al*., 1997) assuming Hardy-Weinberg equilibrium. Hierarchical analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) was performed as described by Huff *et al*. (1993) with ARLEQUIN software (V. 2.0). The populations were divided in to two groups as *Casuarina* and *Allocasuarina* species and the number of permutations for significance testing was set as 1023 for all analysis.

RESULTS AND DISCUSSION

Morphological analysis: Totally 18 morphological characters were recorded from 46 individuals from three *Casuarina* and two *Allocasuarina* species. The morphological traits showed significant differences among the five species for all the examined characters (Table 3) while it did not distinctly differentiate the two

Table 3: List of morphological (physiologically and economically important) characters used to study relationship among the individuals of *Casuarina and Allocasuarina* species

Morphological character	Average	Scored as "0"	Scored as "1"
Plant height (m)	2.92	<Average	>Average
Collar diameter (mm)	36.29	<Average	>Average
Girth at Breast Height - GBH (mm)	14.36	<Average	>Average
Stem form	-	Bend	Straight
Crown shape	-	Open	Pyramid
Number of permanent Branches	18.48	>Average	<Average
Branch Angle		>60°	<60°
Branch length (cm)	135.34	>Average	<Average
Needle length (cm)	34.73	>Average	<Average
Thickness of needle (mm)	1.04	>Average	<Average
Number of nodes per needle	50.82	>Average	<Average
Self-pruning	-	Absent	Present
Bark surface	-	Rough	Smooth
Bark ring	-	Present	Absent
Disease attack	-	Present	Absent
Number of teeth per node	12.83	>Average	<Average
Teeth length (mm)	2.51	>Average	<Average
Internodal length (cm)	0.86	>Average	<Average

genera. The characters like needle length, needle thickness, number of teeth per node, length of internodes and teeth length were similar between *C. equisetifolia* and *A. littoralis* and between *C. junghuhniana* and *A. huegeliana*. The significant parameters differentiating *C. junghuhniana* from other *Casuarina* species (*C. equisetifolia* and *C. glauca)* were plant height, collar diameter, girth at breast height, needle thickness, teeth length, nodes per needles and teeth per node.

Nei's similarity and distances between the three *Casuarina* and two *Allocasuarina* species populations are shown in Table 4. Maximum similarity (0.887) was observed between the species *C. junghuhniana* and *A. huegeliana*, whereas the diversity was found to be high (0.625) between *A. littoralis* and *A. huegeliana* as depicted in the dendrogram. The mean values for the 18 morphological characters (11 quantitative and 7 qualitative) are presented in Table 5. A wide variation was observed in all the five species and differed from each other in one or more individual characters. This confirmed that all the species were morphologically distinct according to NTSYS pc (Fig. 1) as was expected.

The Dendrogram generated based on the morphometric data showed two main clusters between the *Casuarina* and *Allocasuarina* species, one with *C. equisetifolia*, *C. glauca* and *A. littoralis* forming a single group and an another cluster with *C. junghuhniana* and *A. huegeliana* (Fig. 1). Among the populations, maximum and minimum distance was observed in *C. junghuhniana* and *A. littoralis* species respectively.

Table 4: Nei's measures of genetic similarity (above diagonal) and genetic distance (below diagonal) between the species populations of *Casuariuna and Allocasuarina* using

Species	*C. equisetifolia*	*C. glauca*	*A. huegeliana*	*C. junghuhniana*	*A. littoralis*
C. equisetifolia	-	0.581	0.559	0.650	0.704
C. glauca	0.542	-	0.750	0.768	0.576
A. huegeliana	0.582	0.288	-	0.887	0.374
C. junghuhniana	0.430	0.264	0.120	-	0.475
A. littoralis	0.351	0.552	0.983	0.745	-

Table 5: Basic Statistics for eleven morphological parameters analyzed in *Casuarina* and *Allocasuarina* species

	C. equisetifolia		*C. glauca*		*C. junghuhniana*		*A. heugliana*		*A. littoralis*	
Character	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Plant height (m)	3.6	0.6	1.7	0.4	2.9	0.5	1.9	0.3	3.6	0.4
Collar diameter (mm)	46.5	9.5	18.5	4.6	39.0	6.0	29.6	6.1	40.2	4.6
Girth at breast height (mm)	22.9	6.5	4.5	1.2	13.6	6.5	4.2	1.6	17.6	3.3
No. of permanent branches	28.2	6.3	6.0	2.3	17.9	9.3	12.4	4.6	16.2	6.5
Length of branch (cm)	131.2	28.2	117.7	38.3	144.0	33.1	115.9	24.9	110.9	44.2
Length of needle (cm)	25.7	3.7	37.1	13.0	44.2	7.9	45.5	7.8	32.3	4.1
Thickness of needle (cm)	0.9	0.1	1.2	0.1	1.2	0.1	1.3	0.1	0.8	0.1
No. Nodes/needle	49.3	6.3	61.0	13.8	60.0	7.4	45.4	6.4	57.2	6.0
Teeth/node	12.0	0.0	15.0	0.0	15.0	0.0	16.0	0.0	10.0	0.0
Teeth length (mm)	1.5	0.1	2.0	0.2	4.0	0.0	3.3	0.5	2.0	0.0
Internode length (cm)	0.7	0.1	0.8	0.2	0.9	0.1	1.2	0.2	0.7	0.1

SD=Standard Deviation

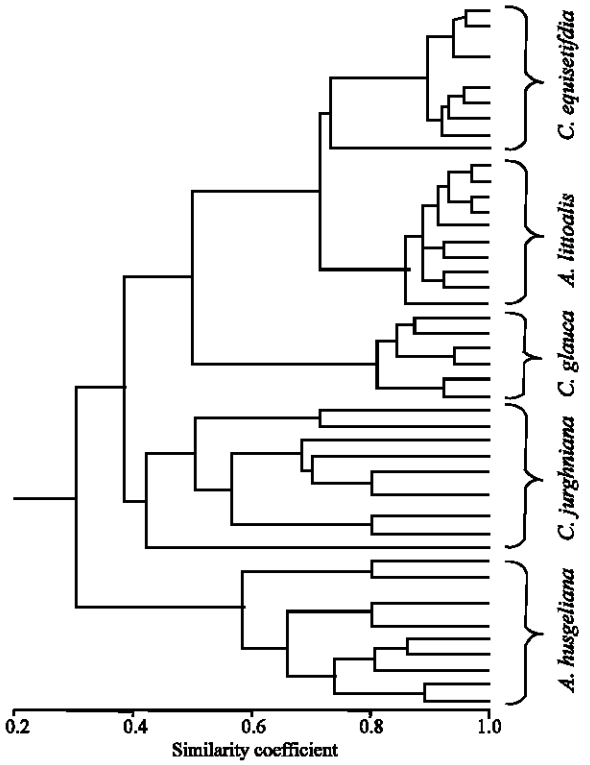

Fig. 1: Dendrogram showing the similarity among the different species of *Casuarina* and *Allocasuarina* based on morphometric data

The first three principal components (PCS), which had eigen value higher than one, explained a total of 66.3% of phenetic variation. The most important characters for Operational Taxonomic Unit differentiation were crown shape, branch angle, branch length, needle length and bark ring; these had high loading on two principal components (PC 2 and PC 3), which explained 16.8 and 10.6% of total variations respectively. The PC 2 was highly influenced by only plant height. The PC 1 was highly influenced by stem form, number of permanent branches, needle length, needle thickness, self-pruning character, number of teeth per node, teeth length and Internodal length, which accounted for 38.8% of total

Table 6: Principal component analysis loadings, Eigen values and percentage of variance for the first three components obtained from morphological characters of *Casuarina* and *Allocasuarina* species

Character	PC-1	PC-2	PC-3
Plant height	0.49	0.80	0.01
Collar diameter	-0.21	0.37	0.15
Girth at breast height	0.02	0.33	0.68
Stem form	0.72	0.49	-0.25
Crown shape	0.55	-0.50	-0.36
Number of permanent branches	0.72	0.49	-0.25
Branch angle	0.30	-0.03	-0.24
Branch length	0.68	-0.44	-0.17
Needle length	0.80	-0.29	-0.23
Thickness of needle	0.77	0.06	0.13
Number of nodes per needle	0.63	-0.09	0.52
Self-pruning	0.91	-0.03	0.18
Bark surface	-0.27	-0.32	0.64
Bark ring	0.38	-0.70	-0.10
Disease incidence	-0.04	0.68	-0.36
Number of teeth per node	0.78	0.01	0.17
Teeth length	0.93	-0.16	0.14
Internodal length	0.81	0.20	0.35
Eigen value	6.98	3.03	1.92
Accumulated variation (%)	38.82	55.65	66.23
Percentage of variance	38.82	16.83	10.64

Table 7: Nei's measures of genetic similarity (above diagonal) and genetic distance (below diagonal) between the species populations of *Casuariuna and Allocasuarina* using ISSR markers

	C. equisetifolia	*C. glauca*	*A. huegeliana*	*C. junghuhniana*	*A. littoralis*
C. equisetifolia	-	0.7585	0.6577	0.6271	0.6306
C. glauca	0.2764	-	0.5471	0.5230	0.5809
A. huegeliana	0.4190	0.6032	-	0.5873	0.5168
C. junghuhniana	0.4666	0.6481	0.5323	-	0.5522
A. littoralis	0.4611	0.5431	0.6601	0.5938	-

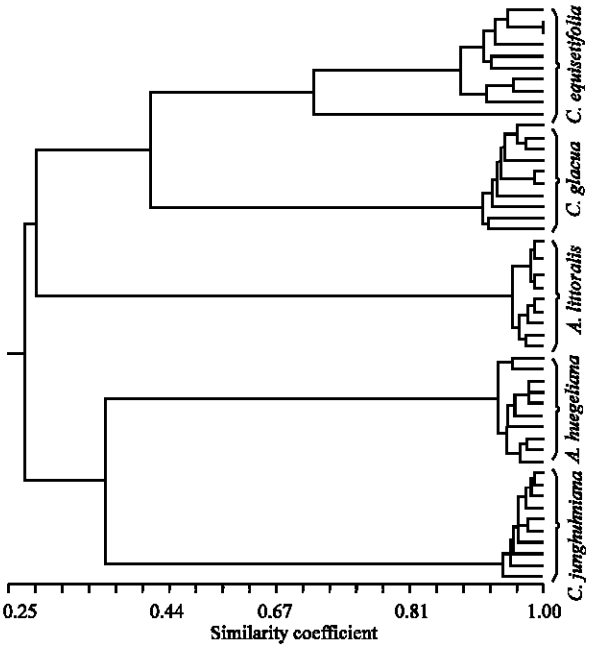

Fig. 2: Dendrogram showing genetic relationship of *Casuarina* and *Allocasuarina* species using ISSR markers

variation (Table 6). All the three principal components analysis were able to differentiate *Casuarina* and two *Allocasuarina* species.

Earlier studies on use of morphological traits to study genetic divergence are limited to the species of *C. equisetifolia* wherein genetic divergence among clones of *C. equisetifolia* were estimated using parameters like height, diameter at breast height, straight and clear main bole, self pruning capacity and non incidence of disease and pests (Kumar and Gurumurthi, 2000). Similarly Barthwal *et al.* (2001) used number of deciduous branchlet, number of nodes per banchlet, number of leaf teeth per node, thickness of deciduous branchlet, internode length, length of needles and bark characters for clonal description of *C. equisetifolia*. In the present study, similar morphological traits differentiated the species of *Casuarina* and *Allocasuarina* and the PCO plots revealed the influence of individual traits on diversity and generated diversity estimates between the species.

Molecular analysis: Seven ISSR primers amplified a total of 226 ISSR loci with size ranging from 220 to 1140 bp in all five species (Fig. 3). Among the species studied, maximum genetic diversity in terms of per cent polymorphic loci was observed in *C. equisetifolia* (18.55%), while the minimum was in *A. littoralis* (4.84%). Maximum similarity index (0.759) was observed between the species *C. equisetifolia* and *C. glauca*, whereas the diversity was found to be high (0.660) between *A. littoralis* and *A. huegeliana* (Table 7) as depicted in the dendrogram (Fig. 2). A considerable genetic relatedness was also found between the populations of *C. junghuhniana* and *A. huegeliana*.

The dendrogram was constructed by cluster analyses using the Nei and Li (1979) similarity matrix for three *Casuarina* and two *Allocasuarina* species. It showed the occurrence of narrow genetic diversity among the individuals of all the *Casuarina* and *Allocasuarina* species, especially in *A. littoralis*. *A. huegeliana* and *A. littoralis* species grouped separately while *C. equisetifolia* and *C. glauca* grouped together. Analysis of Molecular Variance (AMOVA) revealed that the percentages of variance attributable to variation among and within species were 92.69 ($p = 0.10264$) and 8.75 ($p >= 0.50538 \pm 0.01574$), respectively. Among groups, variation components value was negative, -1.44 ($p > 0.40274$). This explained the absence of a genetic structure between the two genera for the targeted loci.

The genetic relatedness of *C. equisetifolia* and *C. glauca* revealed from the present study is in the agreement with observation made by Ho *et al.* (2002) where in natural hybrids between the two species was reported. Yasodha *et al.* (2004) also revealed the genetic relationship of *C. equisetifolia* and *C. glauca*. Earlier studies depicting the phylogenetic relationship between 76 species from four genera within family Casuarinaceae using *matK* gene sequence revealed the distinctness of the two genera *Allocasuarina* and *Casuarina* (Steane *et al.*, 2003). Similarly genetic analyses of 11 species of casuarinas using ISSR markers distinctly grouped the two genera separately (Yasodha *et al.*, 2004). However, in both the above studies, *C. junghuhniana* was not included since they were exclusively conducted on Australian species while the seed source of *C. junghuhniana* used in the present investigation is from Indonesia (Table 1). The present study did not distinctly

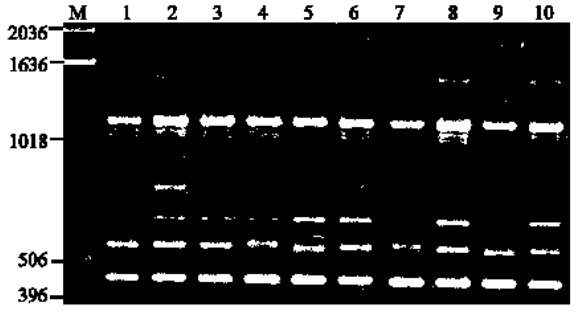

Fig. 3: ISSR profile of *Casuarina junghuniana* populations, with primer TA $(CAG)_4$

group *Casuarina* and *Allocasuarina* using both morphological and molecular data and revealed a significant relationship between *C. junghuhniana* and *A. huegeliana* suggesting that more intensive research using suitable primers, floral characters and inclusion of larger number of individuals for analysis is required to reveal the genetic distinctness of this species.

However, the present study revealed that diversity estimation using conventional morphological parameters (after careful selection of distinct traits) and recent techniques of DNA markers can be used in combination to generate reliable data for utilization in tree breeding and improvement programs.

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