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Genetic diversity, origination and extinction analysis in *Casuarina equisetifolia* using RAPD markers

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ABSTRACT

Development of molecular markers for various desirable traits related to genetic diversity, origination, extinction, conservation, etc hold great potential for crop improvement of Casuarina equisetifolia L. This work focuses on the analysis of genetic diversity, origination and extinction of 24 clones of C. equisetifolia. Clones were collected from Kakinada, Srikakulam, Visakhapatnam and Vijayanagaram, Andrapradsh, India. Total genomic DNA was amplified with random oligonucleotide primers using PCR. The polymorphic bands were detected on agarose gels. Each random primer used in this study produced distinct bands. These were used for the final analysis. Six primers (OPM-05, OPM-13, OPB-18, OPE-06, OPE-07 and OPE-08) yielded 39 scorable bands. Three primers (OPM-05, OPE-06 and OPE-08) generated 100% polymorphic bands and primers OPM-13 (83.3%), OPE-20 (71.4%) and OPE-06 (83.3%) also generated more polymorphic bands. Only primer OPE-20 alone generated less polymorphic bands of 71.4%. These bands were then constructed using the RAPD distance past v 2.02 package. Following this Start method v 2.02 was used to generate a genetic diversity, origination and extinction analysis in between various clones of C. equisetifolia. The genetic diversity range varied from 10 to 18 points, origination from 0 to 4 and extinction from 0 to 2. The highest genetic diversity value of 18 points was recorded in Visakhapatnam clones (APVSP-16, 18, 22 and 23); while the lowest value was observed in Vijayanagaram clones (APVJM-35 and 39). The result provides valid guidelines for collection, conservation and characterization of C. equisetifolia genetic resources.

Key words: RAPD, *Casuarina equisetifolia*, genetic diversity, origination, Past.

***Abbreviations**: RAPD- Random amplified polymorphic DNA, PCR-Polymerase chain reaction, CTAB-Cetyl Trimethyl Ammonium Bromide.*

INTRODUCTION

The Casuarinaceae Family is commonly known as casuarinas. It is a multipurpose tree, widely grown in tropical and subtropical countries for fuel wood, pulp, charcoal, fencing material, buffer zones at beaches and poles [1]. They are also planted for reforestation [1, 2] and to increase the soil fertility through nitrogen fixation [3]. Currently casuarinas are grouped under four genera [8], which encompass over 90 species [4].

Casuarina (2n = 18) comprises about 17 species distributed throughout Southeast Asia and Australia [5]. They are considered to be the nearest relatives of the genus *Casuarina* since they share many common morphological

features. Phylogenetic and taxonomic relationships within the species of casuarinas have been studied using the size and number of chromosomes, pattern of geographical distribution [6, 7] and diversification in the morphological characteristics [8] to distinguish the members of *Casuarina*. Recently, rbcL and plastid matK sequences were used for the genetic analysis of Casuarinaceae [9].

Although about 15 species of *Casuarina* are recognized for multiple utilization, only *C. equisetifolia* is cultivated widely in many countries [5, 10, 11] accounting for about 1.4% of tree plantations of the casuarinas (FAO 1995, [12]). 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 utilization of diversity requires genetic characterization 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* [13]. Genetic variation at the population level has been studied using RAPD markers in *C. equisetifolia* [14]. RAPD variation in casuarinas of Taiwan has revealed that most plants in Taiwan were closely related but not typical of *C. equisetifolia* indicating introgressive hybridisation among *C. equisetifolia*, *C. glauca* and *C. cunninghamiana* [15]. The evaluation of genetic diversity would promote the efficient use of genetic variations in the breeding program [16, 38]. Inbreeding depression has occurred in *Lentinula edodes* [17] and so the evaluation of genetic diversity between breeding materials takes on additional importance [39, 40].

Among many different types of DNA markers, the markers based on Random Amplified Polymorphic DNA (RAPD) provide a co-dominant, highly reproducible and genetically informative marker system [18, 19, 20, 21, and 22]. So the present work was taken up with the objective of establishing an efficient and reproducible marker system based on RAPD-PCR for the identification, characterization, genetic diversity, conservation, clonal selections, and plant breeding of *C. equisetifolia* for forest development.

MATERIALS AND METHODS

2.1 Plant material

Twenty four clones of *Casuarina equisetifolia* were chosen from different plantations in the district of Andhra Pradesh, India (Table 1 and Fig 1). These were used as samples for DNA fingerprinting studies.

2.2 DNA extraction and purification

DNA was isolated from growing tips of juvenile needles of each clone using a modified Cetyl Trimethyl Ammonium Bromide (CTAB) method [23]. DNA concentration and purity were determined by measuring the absorbance of diluted DNA solution at 260 nm and 280 nm.

2.3 RAPD-PCR amplification

The RAPD-PCR was performed according to the method of [24, 23]. The reactions were carried out in 25 µL volume in a tube using ten random decanucleotide primers separately,OPM-02, OPM-05, OPM-13,OPB-04, OPB-12,OPB-15, OPB-18, OPE-06, OPE-07 and OPE-08 (Arbitrary 10-mer primers from Operon Technologies Inc., Alameda, California, USA) [25]. PCR products were resolved on 1.2% agarose gel in 1xTAE buffer. The DNA was visualized and photographed under a UV transilluminator. The sizes of DNA fragments were estimated by comparison with standard ladder (1kb and 100 bp; Bangalore Genei, India).

2.4 Data collection and analysis

The amplification products were scored separately for each primer. The presence or absence of band for each clone was assessed. The binary code 1 for the presence and 0 for absence of the band were used. Only intensely stained polymorphic bands were then constructed using the RAPD distance past v 2.02 package. Following this Start method (diversity curve) v 2.02 was used to generate a genetic diversity, origination and extinction analysis in between various clones of *C. equisetifolia.*

RESULTS AND DISCUSSION

3.1 Marker polymorphism

The random primers produced distinct and Polymorphic profiles in all Twenty four clones of *C. equisetifolia L.* A total of 10 random primers of which 6 produced reproducible polymorphic banding patterns. A total of 39 bands

were scored of which 35 (89.6%) were polymorphic and 4 were monomorphic bands (10.24%). The number of bands generated per primer varied from 6 to 9 and a maximum of 9 bands were observed with primers OPM-05 and OPE-08 while the minimum of 6 bands were generated by the primers OPM-13, OPM-06 and OPM-07. The primer OPB-20 also generated 7 bands. The size of the amplified products varied from 900 to 2,900bp in different primers (OPM-05 (1000bp-2500bp), OPM-13 (900bp-2500bp), OPB-20 (1200bp-2500bp), OPE-06 (1500bp-2900bp), OPE-07 (1350bp-2500bp), and OPE-08 (1250bp-2500bp)). The minimum size (900bp) of the amplified products was from primer OPM-13 and maximum size (2900bp) of the amplified products was from primer OPE-08. Three primers (OPM-05, OPE-06 and OPE-08) generated 100% polymorphic bands and primers OPM-13 (83.3%), OPE-20 (71.4%) and OPE-06 (83.3%) also generated higher polymorphic bands. Only primer OPE-20 alone generated less polymorphic bands of 71.4% [26, 27]. The high level of polymorphism detected by the RAPD markers in this study is comparable with the other perennial species [28]. Similar levels of polymorphic bands were generated in these studies [14, 15, and 29] by the same primers. Similar approach has been successfully used for molecular diagnosis of species and cultivars by many workers [9, 23, 30 and 31].

Table 1: List of *Casuarina equisetifolia* clones and details of collection sites

S.No	CLONAL IDENTITY	Location
1	APKKD-1	Kakinada
2	APKKD-3	Kakinada
3	APPKD-4	Kakinada
4	APPKD-5	Kakinada
5	APPKD-6	Kakinada
6	APPKD-7	Kakinada
7	APPKD-9	Kakinada
8	APPKD-10	Kakinada
9	APPKD-11	Kakinada
10	APPKD-12	Kakinada
11	APVSP-14	Visakhapatnam
12	APVSP-15	Visakhapatnam
13	APVSP-16	Visakhapatnam
14	APVSP-18	Visakhapatnam
15	APVSP-22	Visakhapatnam
16	APVSP-23	Visakhapatnam
17	APSKLM-25	Srikakulam
18	APSKLM-26	Srikakulam
19	APSKLM-27	Srikakulam
20	APVJM-31	Vijayanagaram
21	APVJM-32	Vijayanagaram
22	APVJM-33	Vijayanagaram
23	APVJM-35	Vijayanagaram
24	APVJM-39	Vijayanagaram

Note: All the clones were collected in Andrapradsh- India.

3.2 Genetic diversity

Past (Start- Diversity curve) genetic diversity range varied from 10 to 18 points. The highest value of 18 points was recorded in Visakhapatnam clones APVSP-16, APVSP-18, APVSP-22 and APVSP-23 and Srikakulam clone APSKLM-25, while the lowest value 10 points was observed in Vijayanagaram clones APVJM-35 and APVJM-39. The value of other clones was also observed. Kakinada APKKD-1, 11points; APKKD-3, APKKD-4 and APVJM-33, 14 points; APKKD-5, APKKD-6 and APVJM-32, 15 points; APKKD-7, APKKD-9, APKKD-10, APKKD-11, APKKD-12, 16 points; APVSP-14, APVSP-15 and APVJM-31, 16 points: APSKLM-26 and APSKLM-27, 17 points (Fig 1). Similar wide range in genetic similarity values were observed in many other perennial species [32, 33] and genetic similarity values were observed on seedling growth and seed weight within *C. equisetifolia* species [23]. Seventy individual samples of *C. equisetifolia* growing along the northern coast of Senegal were studied using 160 random primers; they generated 1396 reproducible bands and 61 polymorphic bands. The results showed a narrow genetic variation among (4.36%) and within (5.90%) *C. equisetifolia* subsp *equisetifolia* and *C. equisetifolia* subsp *incana* plantation sites respectively [29]. In *C. equisetifolia*, 456 individual samples were evaluated to assess the genetic diversity and to identify hybridization in *Casuarina equisetifolia* grown in Taiwan and 11 primers were used; 81 polymorphic bands were scored in this study. The average Nei's gene diversity of *Casuarina* grown in Taiwan (0.198) was significantly higher ($p<0.001$) than those of native provenances of *C. equisetifolia* [14]. RAPD markers were also used for the identification of genetic diversity in *C. equisetifolia* of 142 individual samples

belonging to 12 native accessions grown in an international Provenance trial garden in Taiwan [15]. RAPD markers were used in *C. equisetifolia* to characterize closely related genotypes [31].

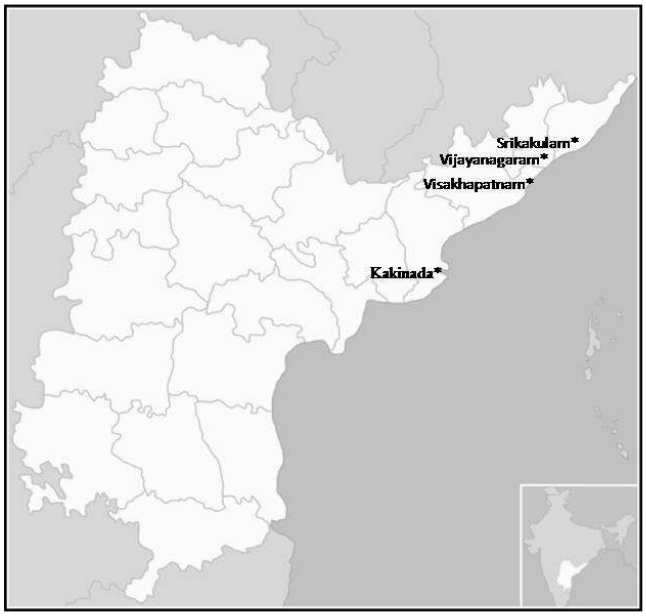

Figure1. Locations of sampling sites for populations of *Casuarina equisetifolia* clones in Andhra Pradesh, India

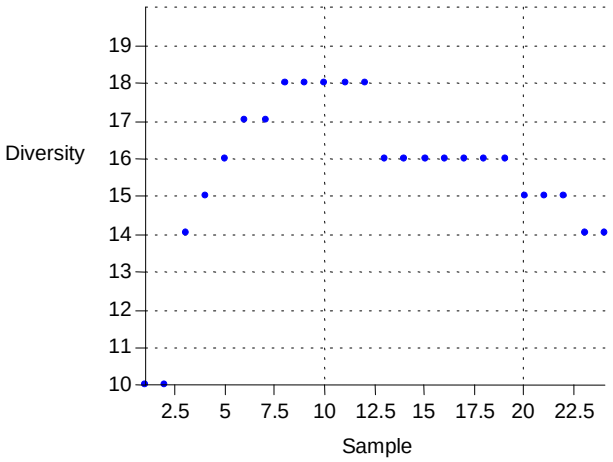

Figure 2.Diversity curve of *Casuarina equisetifolia* clones

3.3 Origination

Origination gives an idea about the origin of the clone. Origin is main reason for genetic diversity within clones and species. Origination points were observed from 0 to 4. The highest origination point 4 was observed in Vijayanagaram clone APVJM-33, while the lowest origination point 1 was observed in Visakhapatnam clones APSKLM-25, APSKLM-26 and APSKLM-27 and Vijayanagaram clones APVJM-31 and APVJM-32. Origination point for other clones was zero. Origination point gives the data about the origin of the clones. Point 4 means that the clones moved four times from one place to another; and one point means that the clones moved one time; the zero point means clones did not move but grew in same place for long time (Fig 3). Similar wide range of

geographical origin values were observed in *Claviceps purpurea* [34, 41, 42]. Twenty-nine field isolates of the broad host range pathogen *Claviceps purpurea* from various host plants and different geographical origin were compared by RAPD analysis. The RAPD patterns obtained showed considerable diversity; it was even possible to discriminate all strains tested with single primer, indicating an unusual high degree of genetic diversity within this species.

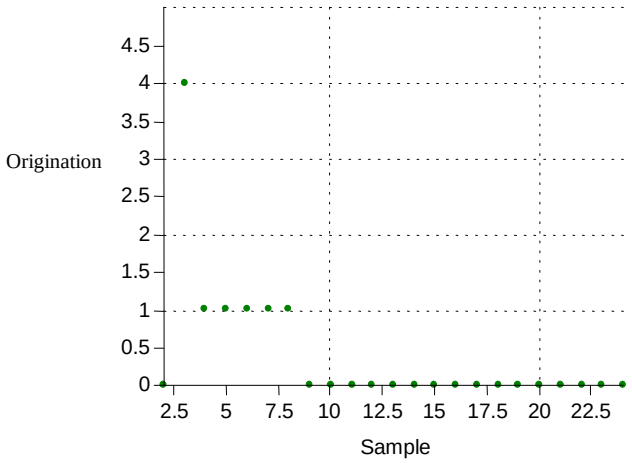

Figure 3.Origination of *Casuarina equisetifolia* clones.

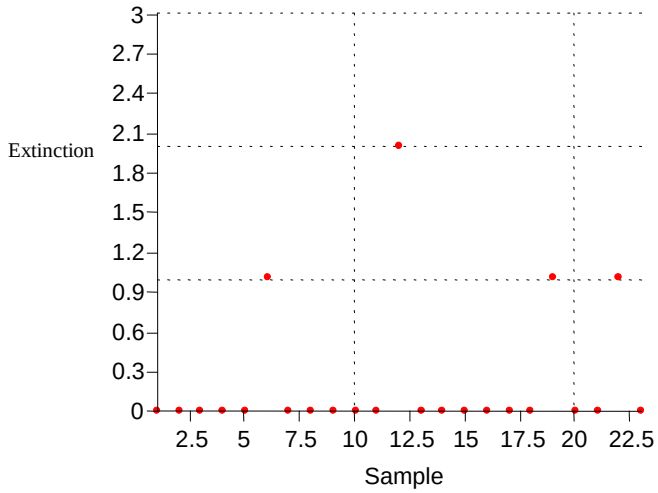

Figure 4.Extinction of *Casuarina equisetifolia* clones.

3.4 Extinction

Extinction is the end of an organism or of a group of organisms (taxon), normally a species. The moment of extinction is generally considered to be the death of the last individual of the species, although the capacity to breed and recover may have been lost before this point. Extinction points were observed from 0 to 2. APVSP-16 showed 2 points, while the APKKD-4, APKKD-7 and APSKL M-27 showed 1 point and other clones showed zero point. Two point is dangerous due to clone is 20% end stage and 1 point also 10% dangerous. Extinction Points gives data about life cycle of the clones, 4 clones were 20% to 10% at end stage of the life among the 24 clones. Clones need to be conserved for plant breeding and their characteristics (Fig 4). Adrian Escudero [35] reported that spatial analysis of genetic diversity can be used as a tool for plant conservation. The existence of spatial genetic structures leads the way to discuss the environmental and biological factors responsible for them. Ibrahim [36] discussed in review article Molecular markers play an important role in estimating the relatedness between the individuals by comparing

the genotypes at a number of polymorphic loci. In tree improvement programs, selection of parents is traditionally done through morphological observations. Progeny tests are conducted to assess the genetic diversity. So the evaluation of genetic diversity between breeding materials takes on additional importance [37]. With the advent of molecular marker techniques, diversity estimations using DNA markers have become important in designing genetic improvement programs. Further, RAPD-PCR is also useful in fingerprinting and characterization of accessions and identification of cultivars and varieties. The present study revealed the genetic diversity, origination and extinction within the clones of *Casuarina* species belonging to the family Casuarinaceae using RAPD marker.

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