Analysis of genetic relationship in superior individuals of *Casuarina equisetifolia* L. using ISSR markers

RR Rasmi¹,*, Daniel Arjuna²,*, Shanthi Arunachalam³, Suguna Paulraj²

¹ Biotechnology, PSG College of Arts and Science, Coimbatore, India
² Plant Biotechnology, Institute of Forest Genetics and Tree Breeding, Coimbatore, India

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Abstract

Inter simple sequence repeat (ISSR) markers were used to assess genetic variation and relationships among the ten superior performing selections of *C. equisetifolia* L. A total of 127 loci were generated by nine ISSR primers (two to three nucleotide repeats anchored with one or two nucleotides at the 3’ or 5’ region), 89 (70 %) were polymorphic. The ISSR analysis also provided information on the frequency of various simple sequence repeats in the *C. equisetifolia* genome. The di-nucleotide repeats were more common, among which (GA)n repeat motif amplified relatively higher number of bands with an average of 14.5±2.38. A higher level of genetic variation was identified among the ten superior performing selections (Nei’s gene diversity, 0.4094; Shannon’s index, 0.5945). The ISSR fragments indicated significant polymorphism and genetic diversity among the ten superior performing selections. An unweighted pair group method with arithmetic mean (UPGMA) dendrogram showed that the ten selections were mainly clustered into two groups, a conclusion further supported by principle coordinate analysis. Based on these results, a management and breeding program for these ten superior selections were proposed.

Keywords: *Casuarina equisetifolia*; genetic relationship; genetic diversity; ISSR-PCR.

INTRODUCTION

*Casuarina equisetifolia* L. is a multi-purpose tree species belonging to the family Casuarinaceae. It is a native of Australia that has naturalized most coastal regions in the tropics and known to grow 20–24 meters in height and 60–80 cm in girth. It is an excellent resource for minor timber, fuel wood, charcoal, fodder and scaffolding. Its fast growth, adaptability to high density planting, drought resilient nature and nitrogen fixing ability has made it a popular choice in farm and agro-forestry (Nagarajan et al., 2006). In India, *C. equisetifolia* is highly preferred for paper pulping and commercial cultivation exceeds thousands of hectares every year (Nicodemus et al., 2001). International provenance trials were established in 20 tropical and subtropical countries for evaluating variations existing within the species (Pinyopusarerk et al., 2004). Breeding programs were established through selection of best provenances and breeding populations (Nicodemus et al., 2001). DNA markers are regarded as the best tools to accelerate breeding in tree species and marker-assisted breeding have been reported for many tropical trees (Ho et al., 2002). DNA markers have been replacing or complementing traditional morphological and agronomic characterization, since they are virtually unlimited, cover the whole genome, are not influenced by the environment, and less time consuming. DNA markers such as inter-simple sequence repeat (ISSR) (Zietkiewicz et al., 1994) are widely used in genetic diversity studies because they need no prior DNA sequence information, development costs are low, and laboratory procedures can easily be transferred to any plant species (Barth et al., 2002). The commonly used polymerase chain reaction (PCR) based DNA marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSRs) or microsatellites (Gupta and Varshney, 2000). The major limitations of these methods are low reproducibility of RAPD, high cost of AFLP and the need to know the flanking sequences to develop species specific primers for SSR polymorphism (Reddy et al., 2002). ISSR is a technique that overcomes most of these limitations (Wu et al., 1994; Zietkiewicz et al., 1994). The ISSR primer is composed of microsatellite sequences either unanchored (Wu et al., 1994) or
anchored at the 5’ or 3’ end by two or four arbitrary, often degenerate nucleotides (Zietkiewicz et al., 1994). The addition of a different base at the 5’ or 3’ end renders their binding sites more specific and reproducible (Barth et al., 2002). The sequence between the two binding sites in opposite orientation within suitable distance is amplified, and indels within this region and loss or gain of binding sites are detected as band polymorphism. The present study reports the use of ISSR markers for assessment of genetic relationship and DNA polymorphism among the ten superior performing selections of *C. equisetifolia* and also shows the suitability of 5’ anchored and 3’ anchored primers in generating polymorphic loci.

**MATERIALS AND METHODS**

**Plant material and DNA extraction**

The *C. equisetifolia* trees were selected from a provenance trial at Pondicherry and a clonal hedge orchard at Coimbatore (Table 1 [Supplementary data]). The trial at Pondicherry comprises thirty five populations that include natural provenances and local landraces. It is the first trial in the country tested in a coastal zone (Nicodemus et al., 2001). The clonal hedge orchard at Coimbatore comprises of more than 120 trees. Among these two locations 10 superior performers were selected based on their silvicultural characteristics (data not shown). These 10 selections (called ‘clones’ henceforth, solely to indicate the mode of propagation from the selected plants) were chosen for ISSR analysis.

The extreme growing needle tips (1 g) were collected and ground using liquid nitrogen in pre-chilled mortar with a pestle to a fine powder and the genomic DNA was extracted using CTAB based modified method (Daniel and Shanthi et al., 2010). To remove polyphenols and polysaccharides, 1% PVP was added to the extraction buffer. An additional washing step with cold ethanol was included for removal of remaining impurities. Integrity and quantity of the extracted DNA were estimated spectrophotometrically and visually verified on 1% agarose gel.

**ISSR amplification**

After a preliminary screening with 35 ISSR primers, 9 primers were selected based on more number of polymorphic bands. DNA amplification (Zietkiewicz et al., 1994) was carried out in 10 μl reaction volume containing 30 ng genomic DNA, 10× Taq polymerase buffer, 10mM of dNTPs, 2.5 mM MgCl2, 0.3 U of Taq DNA polymerase (Bangalore Genei), and 10μM of ISSR primer (Table 2 [Supplementary data]) in a thermal cycler (Palmcycler, Corbett Research, Australia). Amplification cycle consisted of an initial 3 min denaturation at 94 ºC, 35 cycles for 30 sec at 94 ºC, 30 sec at 58 ºC, 1 min at 72 ºC, and final extension step for 10 min at 72 ºC. The amplification products of each reaction mixture along with 1× TBE loading buffer were size fractionated by electrophoresis on a 2 % Agarose gel with 0.1% ethidium bromide and visualized on UV transilluminator and documented using Alpha Imager (Alpha Innotech Corporation, USA). All experiments were duplicated.

**Results**

**Distribution of microsatellite motifs in *C. equisetifolia* genome**

The frequency and distribution of different microsatellite motifs in the *C. equisetifolia* genome were analyzed using nine primers. Such information could be useful in designing the experiments for
microsatellite capture in *C. equisetifolia*. The average number of bands produced by 5′ and 3′ anchored primers were 13.8±1.7 and 14.5±2.3 respectively. The di-nucleotide repeat motif based primers revealed a total of 13.6±0.89 bands and tri-nucleotide produced a total of 13.6±2.30 bands. Among the di-nucleotide repeats, the (GA) in repeat motifs amplified more number of bands with an average of 14.5±2.38. Amplification with the primers based on UBC836 of 3’ anchored (GA)n repeats produced maximum of 18 loci when compared to other primers (Fig. 1).

**ISSR band pattern and genetic analysis**

Nine primers that could amplify reproducible and clear amplification products were selected in this study. Six of the nine primers were dinucleotide repeats. The primer that was based on the poly (CA) motif (R(CA)7) produced more percentage of polymorphic bands (85 %) than other primers (Table 2). The nine primers produced 127 loci across ten different clones studied, of which 89 were polymorphic. The number of bands varied from 11 (RA(GCT)T6) to 18 (UBC836), and 124 size ranged from 100 to 2,000 bp. The average number of bands and polymorphic bands per primer was 14.11 and 9.88, respectively. The percentage polymorphism ranged from 53% (TA(CAG)4) to 85% (R(CA)7), with an average of 70% across all the samples. The ISSR bands were scored for presence (1) or absence (0) among the clones and used for the UPGMA cluster analysis. A dendrogram based on UPGMA analysis with ISSR data was produced, with Jaccard’s dissimilarity coefficient from 0.1340 to 0.4495 (Table 3 [Supplementary data]). The Genetic diversity of every individual was evaluated with Shannon’s index of diversity (I) and Nei’s genetic diversity (H). The result showed that Shannon’s index of diversity was in range of 0.5945±0.0208, and Nei’s genetic diversity in the range of 0.4094±0.0183 (Table 4 [Supplementary data]). The result of this study suggests that there is substantial genetic variation and polymorphism across the clones of *C. equisetifolia*.

Cluster analysis of ISSR based on Jaccard’s dissimilarity coefficients using UPGMA identified two main groups (Fig 2). Group I included eight clones (TNVM2, TNPK1, TNRM8, TNPPB1, TNPP3, TNPT8, TNPT15 and TNPT6), Group II included two clones (PY131 and PY119), which were genetically more isolated (Fig 2). The two groups associated according to their regions. PY131 & PY119 of Pondicherry (PY131 and PY119), which were genetically more closely linked. The two Tamil Nadu clones from Coimbatore trial (TNRM8 & TNPB1) were also closely linked. The clones of group I were subdivided into two subgroups at the genetic distance of 0.36. The first subgroup was formed by five clones (TNVM2, TNPK1, TNRM8, TNPPB1, & TNPP3) and second subgroup was formed by three clones (TNPT8, TNPT15 & TNPT6). The UPGMA results were supported by the two-dimensional pattern established from the principal coordinate analysis (Fig 3) of ISSR data. The first three coordinates accounted for 73.52 % of the total variation. The relationships among the clones in this figure were supported by the result of cluster analysis. The PY119 & PY131 clones of Pondicherry was the most isolated clones. The remaining clones were divided into two clusters similar to the result of cluster analysis.

**DISCUSSION**

The present study found high genetic diversity at the molecular level among *C. equisetifolia* clones (P = 70%). This result is in correlation with the report of Yasodha et al. (2004). The amplification of ISSR markers was consistent across DNA samples of superior clones of *C. equisetifolia*. Over 99% of the scoreable ISSR fragments were reproducible in the present experiment. The high reproducibility of ISSR markers may be due to the use of longer primers, allowing for more stringent (higher) annealing temperatures than those used for RAPD (Kojima et al., 1998). The ratio of polymorphic loci is 70 % for 127 amplification products with 9 primers. Each primer averaged 9.8 polymorphic loci. Yasodha et al. (2004) studied 12 clones of *C. equisetifolia* by ISSR and found each primer averaged 8.1 polymorphic loci, which is consistent with our report. Feng et al., (2006) studied *Pinus koraiensis* with 15 ISSR primers, and found an average of 3.6 polymorphic loci, which is significantly lower than found in the present study.

Moreover, the choice of primers used in amplification is critical to demonstrate high polymorphism. All the primers used in our study amplified large numbers of loci, varying from 11 to 18 with higher degree of polymorphism (70%). The results showed that Shannon’s index of diversity was in range of 0.5945±0.0208, and Nei’s genetic diversity in the range of 0.4094±0.0183. The genetic parameters estimated from the ISSR data indicated that there was substantial genetic diversity within the individuals of *C. equisetifolia*. This superiority of ISSR has also been reported earlier in other species (Aga et al., 2005; Boronnikoval et al., 2007; Feng et al., 2006; Fernandez et al., 2002; Feyissa et al., 2007; Yingjuan et al., 2009; Zhao et al., 2007). The inter-simple sequence repeats are regions lying within the microsatellite repeats, have a high capacity to reveal polymorphism, and offer great potential to determine genetic diversity compared with other arbitrary primers, like RAPD (Zietkiewicz et al., 1994).

In general, high levels of genetic diversity are not expected with strictly limited distribution and a small population size. For example, using 11 RAPD primers, no variation was detected among 29 individuals of
Limnium cavanillesii (Palacios and Gonzalez-Candelas, 1997). Nevertheless, ISSR markers used in this study tend to generate higher levels of polymorphism compared with other molecular markers, such as RAPD and AFLP (Deshpande et al., 2001; Ge and Sun, 1999). This shows that small populations or individuals are not always associated with a lack or low levels of genetic variation (Yingjuan et al., 2009). Similar type of results was recently reported in other tree species, Euryodendron excelsum (Yingjuan et al., 2009), Hagenia abyssinica (Feyissa et al., 2007), Pinus koraiensis (Feng et al., 2006).

The study revealed that di-nucleotide repeat motifs were abundant in C. equisetifolia genome. The 3’ anchored (GA)n repeats produced maximum of 18 loci when compared with other primers. The relative abundance of di-nucleotide repeats in Casuarina appears to be similar to the report of Yashoda et al., (2004) in Casuarina species and Awasthi et al., (2004) in Morus species. Moreover, the study also utilized very low quantity (30 ng) of template DNA but able to generate higher level of polymorphism. The study also illustrates the advantages of the ISSR-PCR system for the fingerprinting of C. equisetifolia clones generating higher number of bands per assay using very little quantity of template DNA. The results shows that the use of ISSR markers is more reliable and suitable for molecular profiling of various clones of C. equisetifolia collected from diverse places. These results demonstrated that ISSR assays could generate ideal markers for fingerprinting. Besides the findings of this study, high genetic variation using ISSR markers utilizing low quantity of template DNA was also recorded in Sinojackia dolichocarpa (polymorphic loci 77.99%, heterozygosity 0.2255) (Cao et al., 2006) and Primula interjacens (percentage of polymorphic loci 75.47%, heterozygosity 0.3205) (Xue et al., 2004) based on ISSR data.

In conclusion, it is thus possible to establish a fingerprint reference library of ISSR profile of the ten clones of C. equisetifolia L. Such information will be of use in identification and verification of genotypes in clonal plantations and clonal seed orchards. The advantages of ISSR markers are well documented in agronomic species (Godwin et al., 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 et al., 1998), Coca (Charters and Wilkinson, 2000), Larix (Arcade et al., 2000) and Morus (Awasthi et al., 2004). The clones which showed higher genetic divergence in the present study could also be further used in tree breeding and improvement programs.

References


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