International Journal of Science and Research Archive

eISSN: 2582-8185

Cross Ref DOI: 10.30574/ijsra

Journal homepage: https://ijsra.net/

(RESEARCH ARTICLE)

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Characterization of assembled clones of *Pongamia pinnata* (L.) Pierre for its genetic variability using ISSR

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International Journal of Science and Research Archive, 2022, 07(02), 173–180

Publication history: Received on 05 October 2022; revised on 13 November 2022; accepted on 16 November 2022

Article DOI: https://doi.org/10.30574/ijsra.2022.7.2.0251

Abstract

Genetic diversity of *Pongamia pinnata* within and among 5 populations collected and assembled from two different agroclimatic zones of Tamil Nadu, India. Totally 25 accesions were examined with eight inter simple sequence repeat (ISSR) primers. A total of 120 scorable bands were generated, among which 80% were polymorphic. Jaccard similarity coefficient was calculated for pairwise comparisons. Within all 25 accessions and ranged from 0.30 to 0.88 while average within population similarity ranged from 0.50 to 0.60. Within populations variability was estimated as percentage polymorphic loci (ranging from 50% to 80%). Shannon's information index among two zones were detected. Between and within accessions relatedness was estimated among populations with a principal coordinate analysis and also estimated with cluster analysis (UPGMA). These estimation depicts outcrossing rates both within population and between-population diversity. Molecular marker characterization can however be used to identify the most suitable zone for collection of high polymorphic accessions for tree improvement program with the yield.

Keywords: ISSR; DNA marker; Genetic diversity; *Pongamia pinnata*; Agroclimatic zone

1. Introduction

Pongamia pinnata (L.) Pierre gains most important potential oil yielding tree. It is distributed widely throughout the country, grows in all kinds of soils including degraded and wastelands. It is a nitrogen fixing tree and thereby enhances the soil fertility. The Pungam seed contains about 30 to 40% of oil yield and it is one of the potential biodiesel species. A total of 25 high fruit yielding trees of *P. pinnata* were selected based on morphological features such as number of pods in one foot branch, total pod yield, height of the tree, and GBH and crown area in two agroclimatic zones of Tamil Nadu. Naturally it's occur along coasts and river banks in India, Myanmar, Australia and Philippines. In India, its presence and dispersion of diversity is represented around the country in all types of areas. The variability varies with the change in the altitudinal variation in specific areas. Unique elite phenotypic diversity and variation is observed in traits, like colour of flower, branching pattern, palmate leaf number, leaf colour, pod size and shape etc. when it looked as a cross pollinated species. Its characteristic nature such as cooling shade has framed as an important species of urban forestry. Vegetative propagation through rooted stem cutting, traditional method and could be a viable solution for establishing clonal plantations of *P. pinnata*. A preliminary method for propagation of pongamia *via* juvenile cuttings using auxins and B vitamin was reported (Palanisamy *et al.,* 1998, Karoshi and Hegde, 2002). Many researchers explored the suitability of fatty acid methyl esters (FAMEs) from Pongamia seed oil as a good fuel for diesel engines. This has been used as lamp oil, in leather tanning, in soap making, and as a lubricant for thousands of years. Their toxic chemical content, its color, bitter taste, and disagreeable odour, keep it from being used in cooking, but it is traditionally used in medicine making for treating skin disease and liver disease.

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Many DNA marker systems have been developed to rate genetic diversity in plant species. Commonly used PCR-based marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSRs) or microsatellites (Gupta and Varshney, 2000). Although, sometimes low reproducibility of RAPD and high cost of AFLP, and the need to develop species-specific primers for SSR analysis are major limitations, all of which are overcome by the inter simple sequence repeats (ISSR) technique. ISSR is a PCR-based technique that involves amplification of a DNA segment between two inversely oriented identical microsatellite repeat regions (Reddy et al. 2002). In plants, dinucleotide repeats are most prevalent (Wang et al. 1996) and often used in the primers (Moreno et al. 1998). Long primers (16–25 mer) allow the utilization of high annealing temperatures (45– 60 ℃), which is probably the reason for the improved reproducibility of ISSR compared to RAPD where 10-mer primers are used (Reddy et al. 2002, Xiaoyan Liu et al., 2020).

Conservation and improvement of the ecologically and economically valuable pongamia would have significant effects for agroforestry species in various zones of Tamil Nadu. The objective of this study was to analyse genetic diversity for conservation and genetic improvement of this species.

2. Material and methods

2.1. Plant material

A total of 25 leaf samples from 5 populations were collected from two agroclimatic zones of Tamil Nadu ie. , cauvery delta zone (Thanjavur, Trichy, Nagapttinam) and southern zone (Madurai, Thoothukudi) (Figure 1, 2 & 3). Each population was represented by 5 individual trees.

Figure 1 *Pongamia pinnata* tree

Figure 2 Branch with many fruits

Figure 3 Variation in seeds

2.2. DNA isolation

About 1.0 g of young leaves were harvested from the tip of new shoots on the stem or branches of the trees. Total genomic DNA was isolated from silica gel-dried leaves using the CTAB method (Wang *et al*. 1996) with minor modifications. Quality of the DNA was checked by electrophoresis of the samples on 1% agarose gel and staining with ethidium bromide. DNA concentration was determined by spectrophotometry.

2.3. PCR amplification and electrophoresis

Fifteen primers (IDT) were screened on individuals of different populations and regions, and eight primers that produced clear and polymorphic band pattern were selected for further study.

DNA amplification was carried out in a total volume of 25 µl containing 0.20umol/L primer, 0.28mmol/L dNTPs, 40ng templet DNA, 1.5mmol/L Mg2+, 1.0U Taq DNA polymerase (Bangalore Genei). The water used in the reaction contained 0.1% (v/v) diethyl pyrocarbonate (DEPC) before autoclaving. Amplification was performed using a Thermal Cycler BIORAD® PCR System under the following temperature profiles: an initial denaturation step of 1 min at 94 °C, followed by 40 cycles of 1 min at 94 °C (denaturation) followed by 2 min at 55°C (annealing) at a ramp rate of 0.5 °C s–1 and then 30 s at 72 °C (extension) at a ramp rate of 1.3 °C, this ramp rate was also used to rise to 94 °C. The last cycle was followed by a final extension step of 5 min at 72 °C.

The PCR products were electrophoresed on polyacrylamide gel. Loading buffer (5 µl) was added to each PCR product and 6 µl of the mix was loaded in each slot of the gel. The loading buffer consisted of 20% (w/v) sucrose, 0.05% (w/v) bromophenol blue, 5 M urea, and 1 mM EDTA. A 100 bp ladder was loaded in left and right border slots of the gel. When

the electrophoresis dye reached the strip at the anodal end, the gel was taken off and the PCR products were visualized by using BIORAD image Doc XR+ system.

2.4. Data scoring and analysis

Every ISSR band pattern was measured as an independent locus and polymorphic bands were scored as absent (0) or present (1) for all the 25 individual samples. Considered clearly reproducible bands were scored and differences in band intensity were not considered. Polymorphic bands taken for data analyses. A pair-wise genetic similarity matrix was generated using Jaccard similarity coefficient. A principal coordinate analysis was performed based on similarity for qualitative data (SIMQUAL) for all individuals and a plot was generated using NTSYS-PC version 2.1 (Rohlf, 2000). For population variation, we calculated percentage polymorphic loci, Nei's (1973) gene diversity (HS), average Jaccard similarity coefficient and Shannon's information index (Hpop). All above parameters were then compared using Pearson correlation analysis.

For estimation of between-population genetic variation, total genetic diversity (HT), mean within-population genetic diversity (HS), among populations genetic diversity (DST), and the coefficient of genetic differentiation (GST) were calculated using the expressions HT = HS + DST, and GST = DST/HT (Nei 1977). We also calculated Shannon-index based genetic diversity for the species (Hsp), mean within- population genetic diversity (Hpop), proportion of genetic variation within populations (Hpop/Hsp), and proportion of genetic variation between populations (Hsp– Hpop)/Hsp (King and Schaal 1989).

Similarities among the five populations were quantified with the Jaccard similarity coefficient and visualized using a cluster analysis (un- weighted pair-group method with arithmetic averages, UPGMA) and illustrated in a phenogram using NTSYS-PC version 2.1.

3. Results

The eight ISSR primers produced a total of 120 bands that could be unambiguously scored. Of these bands, 96 (80%) were polymorphic in the 25 *Pongamia pinnata* samples although some bands were polymorphic only in certain populations. The number of polymorphic bands ranged from 7 for primer 870 to 15 for primer 820 with a mean of 10.5 bands per primer, and the size of the bands ranged from 225 to more than 3000 (Fig. 7).

3.1. Within-population variation

Number of polymorphic bands within populations varied from 50 % for Madurai to 87% for Thanjavur, with a mean of 70%. Madurai also had the lowest values for Shannon index-based genetic diversity, 0.31, and Nei's genetic diversity, 0.21. Thoothukudi and Trichy had the highest Shannon index value, 0.50, and the highest Nei's genetic diversity, 0.35. Pearson correlation analyses showed that all three parameters yielded closely correlated estimates of within-population variation, $r = 0.950$, $P < 0.001$. Jaccard similarity index values were, as expected, negatively correlated with the other three parameters, $r = -0.75$, $P < 0.01$.

3.2. Variation among populations

Calculations of total genetic diversity (HT), average within-population diversity (HS), among- population diversity (DST), and coefficient of among-population differentiation (GST) yielded 0.40, 0.30, 0.10 and 0.25, respectively. Corresponding analyses of Shannon's information index for mean genetic variation for all the 120 individual samples (Hsp), average within-population diversity (Hpop), proportion of genetic variation within populations (Hpop-Hsp), and proportion of genetic variation between populations (Hsp–Hpop)/Hsp yielded 0.58, 0.43, 0.74 and 0.26, respectively. Interestingly, the two parameters for estimation of population differentiation, i.e. GST and (Hsp–Hpop)/Hsp, yielded highly similar values; 0.25 and 0.26, respectively. Jaccard similarity coefficient-based pairwise comparisons of the 5 populations showed that Thoothukudi 1 and Madurai 1 are the closest with 0.72, whereas Madurai 2-5, Trichy 1-5 and Thanjavur 1-5 as well as Nagapattinam1-5 are the limited distant populations with a similarity coefficient of 0.65.

3.3. Principal coordinate analysis

Jaccard similarity coefficients for pairwise comparisons among all the 25 individuals ranged from 0.30. A principal coordinate analysis based on the Jaccard values was undertaken; the first principal coordinate accounted for 6.6% of the variation, the second for 5.1% and the third for 4.1% (Fig. 5). Although there was considerable overlapping between samples from different populations and regions, some patterns could still be discerned. Differentiation was found also within these groups. This plot clearly suggests that there is a relationship between geographical locations and the ISSR data.

3.4. Cluster analysis

A UPGMA-based cluster analysis of the 25 sampled populations showed that Madurai 1, Thoothukudi 1 forms one cluster, Remaining all accessions from Madurai 2-5,Thoothukudi 2-5, Nagapattinam 1-5, Thanjavur 1-5, and Trichy 1-5 formed another cluster, thus forms two cluster with overlapping closely (Fig. 4).

3.5. Structure Analysis

Population structure of the 25 germplasm lines was analysed by Bayesian based approach. The estimated membership fractions of 25 accessions for different values of k ranged between 2 and 5 (Fig. 6). This indicated that the entire population can be grouped into two subgroups the differentiation in origin and seasonal differentiation contributed for this clustering.

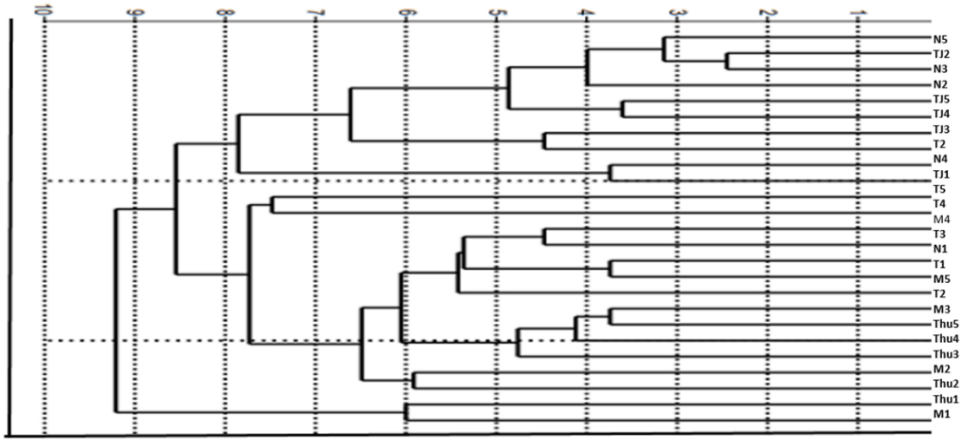

Figure 4 Dendrogram depicting genetic relationship among 25 accessions of *Pongamia pinnata based* on ISSR data using UPGMA

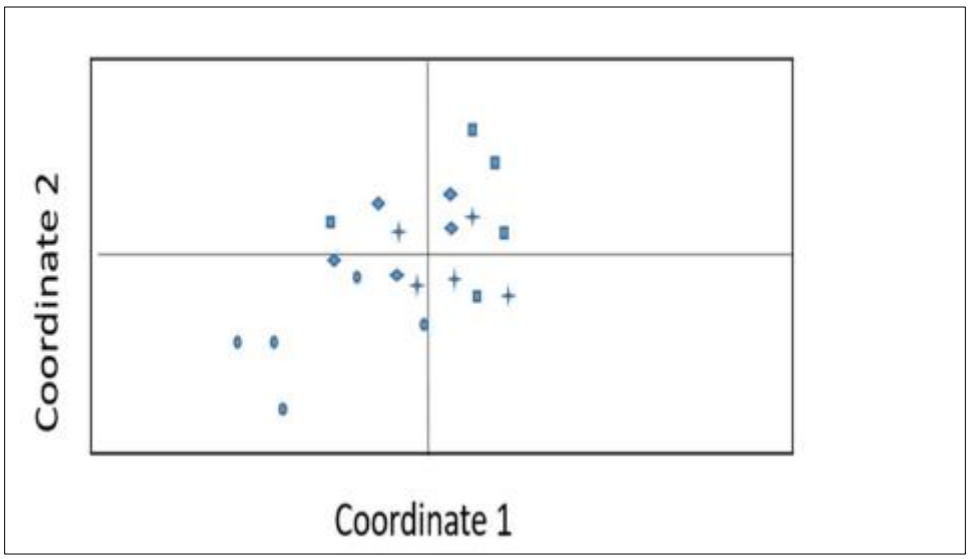

Figure 5 Principle coordinate analysis (PCoA) dots are sampling locations

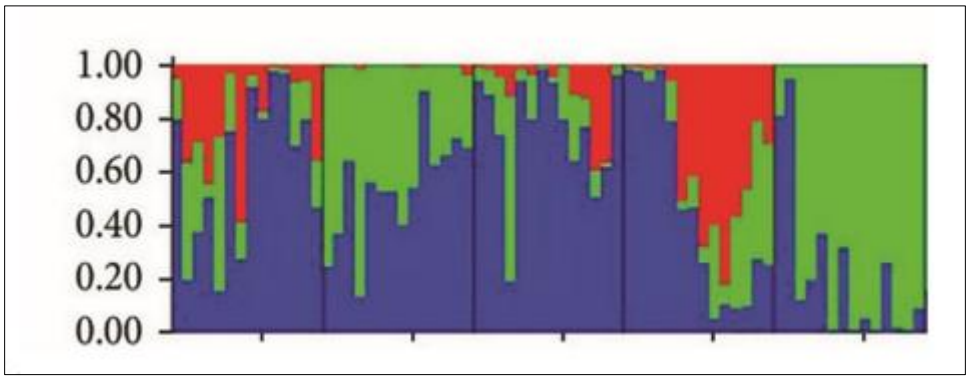

Figure 6 Population structure shows two different overlapping groups of five populations

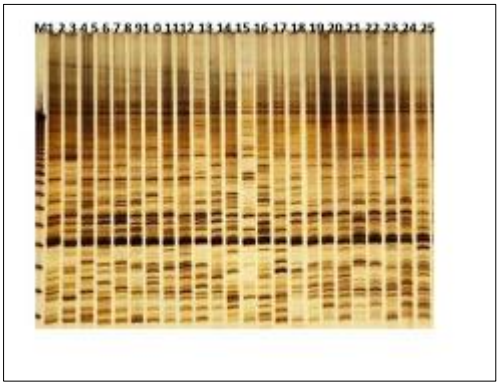

Figure 7 Analysis of genetic diversity in *Pongamia pinnata using* ISSR marker

4. Discussion

Characterization of accessions using molecular markers to find genetic diversity in tree species have become important tools in tree improvement and conservation (Weising et al. 2005). Generally, genetic diversity of a species is strongly associated with phyletic group, life form, geographic range, regional distribution, breeding system, seed dispersal mechanism, mode of reproduction and successional status. Of these para- meters, breeding system appears to be the most important factor, followed (in no particular order) by life form, seed dispersal and successional status (Ni Luh Arpiwi et al., 2019).

The present study reports genetic diversity parameters for the oil yielding tree species *Pongamia pinnata* ISSR-based analysis revealed values for within-population diversity (mean HS = 0.30) that are somewhat higher than the overall value of 0.22 for a set of four ISSR- based studies compiled in (Ni Luh Arpiwi et al., 2019). In this and other compilations (Weising et al. 2005), ISSR-based estimates have been shown to be very similar to estimates based on AFLP (average for 13 studies = 0.23) or RAPD (average for 60 studies = 0.22). For ISSR the values obtained was 0.30 for *Pongamia pinnata,* for long-lived perennials (0.25) that are outcrossed (0.27), dispersed by wind or water (0.27) and belong to late successional species (0.30). In population structure, two sub groups classification has the factor of ecosystem and seasonal variation as the major factors for population structure. Liakat Ali et al. (2011) has substantiated this statement with the reason of the rice subpopulation occupying the largest rice growing region which has a varied environments, ecological conditions and soil type.

For DNA marker-based between-population differentiation, the most commonly reported parameters in the literature are FST (usually calculated using AMOVA) and GST which generally produce highly similar values, and Shannon's index which produces values that vary more (in either direction) from the other two. In our study, *P. pinnata* populations had almost the same value for GST (0.25) and Shannon's index (0.26). In a compilation of ISSR-based studies, 9 studies had an average FST value of 0.35 and 6 studies had an average GST value of 0.34. These values are quite similar to overall

values obtained in studies where ISSR was used instead (0.34 and 0.27, respectively), therefore suggesting that ISSR and RAPD produce comparable results (Nybom 2004; Nilkanta et al., 2017;Ni Luh Arpiwi et al., 2019).

5. Conclusion

Pongamia pinnata is known to show diverse distribution pattern along the various geographical locations of Tamil Nadu. Genetic diversity within populations is also influenced by many factors such as mating system, population size, extended time period with low number of individuals, genetic drift, and gene flow. High genetic diversity within small populations can also be exhibited if reduction of population size had taken place very recently, especially when it occurred within a generation or two for the concerned species. In such cases the surviving individuals are effectively sampled from the populations that have existed before. Molecular markers help us to measure the level of genetic diversity that exists among varieties and accessions which can be utilized in tree improvement programs. Population structure clarifies the relationship between accessions which indicates that germplasm lines varies based on its ecology and also shows higher level of genetic diversity exists within population. The two sub groups classification has the factor of ecosystem and seasonal variation as the major factors for population structure. The existence of significant genetic variation within the populations of *P.pinnata* under study may also be due to sudden reduction in population size in short span of time. This presumption might hold true as more accessible individuals growing in the region have been exploited by locals for construction of houses and other household products. Hence, the results of this study which indicates the genetic diversity of the accessions can be utilized to predict approaches such as association analysis, classical mapping population development; parental line selection in tree improvement programs and hybrid development for exploiting the natural genetic variation exists in this population. The presence of high genetic variation within and among populations of *P.pinnata* emphasizes the necessity of preserving and conserving all the high fruit and oil yielding germplasms for tree improvement.

Compliance with ethical standards

Acknowledgments

The authors are grateful to Institute of Forest Genetics and Tree Breeding (IFGTB) under the Indian Council of Forestry Research and Education (ICFRE) for this diligent guidance of my work.

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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