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Genetic diversity analysis by RAPD markers in candidate plus trees of *Pongamia pinnata*, a promising source of bioenergy

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ABSTRACT

Pongamia pinnata has received much attention in recent years as a source of seed oil that can provide a substitute for diesel fuel. Very little molecular work has been reported on this species. This paper reports our studies on the diversity amongst the CPTs (candidate plus trees) of *P. pinnata* previously identified on the basis of morphometric traits, particularly pod and seed traits. For this RAPD (random amplified polymorphic DNA) markers were used to determine the genetic diversity among 10 genotypes of *P. pinnata* CPTs selected for suitability for energy production. For further investigation, 18 primers generating stable band patterns from 40 tested arbitrary primers were selected. A total of 210 amplification products were obtained of which 10.48% were polymorphic. The genetic similarity index ranged from 0.11 to 0.87. Genetic distance values were used to generate a dendrogram (UPGMA) between the genotypes. The Mantel method used for comparing the similarity matrixes produced correlation coefficients that were statistically significant for the RAPD marker. This genomic analysis allows a cost effective characterization of CPTs of *P. pinnata*. The present investigation supports in future the development of genetic map in *Pongamia* which are a highly useful tool in breeding and may provide information on the inheritance of features crucial for increase seed yield, oil content and the resistance to key insects and pests. Thus this study warrants *Pongamia* producers to realize its full potential and contributes for its sustainable production and improvement.

1. Introduction

The increasing price of petroleum oil products is likely to have serious implications for the automobile industry in the future. This situation has prompted a search for a renewable alternative to the fast depleting reserves of fossil fuel. *Pongamia pinnata* is one of the species that satisfy this requirement [1].

P. pinnata popularly known as "Karanj" is a commercially significant tree species that adapts to wide agro climatic conditions. This legume has attracted the world's attention as a source of sustainable substitutes for petroleum products that can grow in and reclaim marginal lands [2]. The tree is also valued for its medicinal effects [1,3]. The attributes of *Pongamia* make it an ideal biofuel crop as it is easy to grow, has a short generation time, produces large quantities of seed as the plant flowers twice and has a wide genetic base [4]. We have shown on the basis of morphometric (both vegetative and reproductive) traits that considerable diversity exits among the naturally growing populations of *P. pinnata* [5]. This diversity of *P. pinnata* is a very important consideration for the establishment of a biofuel cropping system and for the success of crop breeding programs [4].

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EtBr, ethidium bromide; CPT, candidate plus tree; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; SDS, sodium dodecyl sulphate; TE, tris-EDTA buffer.

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The effectiveness of tree breeding/improvement programmes depends upon the nature and magnitude of existing genetic variability. A systematic germplasm collection, characterization and evaluation programme is essential to identify superior planting material from the existing natural variations. The characters used to evaluate systematic relationships and to discriminate between *Pongamia* individuals are traditionally and primarily morphological characters (quantitatively and qualitatively) that are scored in field and in seed laboratory tests [5,6]. Though such phenotypic evaluations are important, the number of morphological traits is limited and not understood at the gene level. This is because most economic characters are polygenically inherited and their expression is influenced by environmental conditions. The germplasm diversity evaluation has been tremendously empowered by invoking biomolecular analytical techniques like DNA polymorphism profiling, thus facilitating direct and reliable measurements of genetic divergence [7,8]. The DNA based markers significantly shorten the evaluation process and eliminate the problematic influence of external factors. The same DNA marker may also be used in identifying genotypes, determining the degree of genetic similarity and even selecting genotypes that deliver the most desirable attributes (marker assisted selection). For inter and intra-population assessment with limited genetic variability, the molecular markers of choice must be very informative. Although newer techniques like AFLPs, microsatellites (SSR and ISSR) are preferred due to their informativeness, **R**andom **A**mplified **P**olymorphic DNA (RAPD) analysis [9] is still used because of its simplicity, low cost and lower infrastructure requirement [10]. It supports the simultaneous detection of polymorphism at many loci in the entire genome. RAPD is thus a highly effective tool for investigating the genetic diversity of living organisms, and it is definitely a very useful technique at the initial stage of such studies.

Despite the importance of this potential biodiesel legume plant and the availability of appropriate molecular genetic tools, there have been few studies on the genetic structure and variation in *Pongamia* genotypes [11]. No significant research has been undertaken to improve *Pongamia* for the desired traits. Research at the molecular level on this species has lagged behind that of other biodiesel crops such as *Jatropha*. One of the important factors restricting the large scale production and development of high seed and oil yielding varieties/genotypes is that no information is available about genetic diversity in naturally growing populations of *Pongamia* at inter or intra population level**s**. It is, therefore, essential that genetic variability in naturally growing *Pongamia* populations be assayed in the context of the total available genetic diversity for each individual. The data comprise morphological and reproductive traits measured on 50 trees and each of the 50 trees is a different genotype. Candidate plus trees (a CPT is an individual tree possessing superior morphological and reproductive characters than other individual of the same species) of *P. pinnata* identified on the basis of morphometric markers across ten different populations for two consecutive seasons using CROPSTAT [5] inspired us to subject genotypes of *P. pinnata* to RAPD marker analysis.

The objective of this study was, therefore, to evaluate the genetic diversity of candidate plus trees of *P. pinnata*, selected for biodiesel generation purposes, by the use of RAPD markers.

2. Materials and methods

2.1. Plant material

The germplasm used in this investigation of genetic diversity was seeds from CPTs of *P.pinnata* collected from each of the 10 different genotypes from the study site at Sila Forest Range, North Guwahati, Assam (latitude 26°14′6″ N and longitude 91°41′28″E). Fresh seed samples collected during the month of September were stored at −20 °C for all future biomolecular analysis.

2.2. DNA extraction

Total genomic DNA was extracted from seeds collected during the month of September for each of the 10 superior genotypes using a modified SDS protocol [12]. The quality and quantity of the extracted DNA was confirmed to be consistent both spectrophotometrically and by running the extracted DNA on 0.8% agarose gels stained with ethidium bromide (0.5 μg/mL). At least two independent DNA preparations for each genotype were made, and the quantity and quality of DNA samples were estimated.

2.3. RAPD analysis

For RAPD fingerprinting, PCR amplification of the genomic DNA was carried out using 40 arbitrary decamer oligonucleotide primers (Operon Tech, USA). Following preliminary selection, 18 primers generating amplification products with stable banding patterns were selected for the study (Table 1). Each 20 μl of reaction mixture contained 50 ng/μl of template DNA, 1x assay buffer (100 mM Tris sulfonic acid, pH 8.8, 15 mM $MgCl_2$, 500 mM KCl and 0.1% gelatin), 0.2 mM each dNTPs (B'LGenei, India), 5 pM of each primer and 0.5 U of Taq polymerase (B'LGenei, India). The reaction was performed in 0.2 ml microfuge tubes (Dialabs). PCR amplification was carried out in a Mini Thermal Cycler (Applied Biosystems 9700) programmed for 35 cycles. The first amplification cycle consisted of an initial denaturation step of 5 min at 94 °C, followed by 34 cycles of 45 s at 94 °C, annealing for 1 min at 32 °C, and extension at 72 °C for 1 min 30 s. An additional cycle of 5 min at 72 °C was used for primer extension. The amplification products were electrophoresed in 1.5% agarose gels in 0.5x TBE (10x stock contained 0.8 M Tris, 0.8 M boric acid, 0.5 M EDTA). A 1 kb DNA ladder (Life Technologies, USA) was included as a size marker and the amplified products were detected with ethidium bromide staining (0.5 μg/mL). The gels were photographed under a UV transilluminator.

2.4. Data analysis

The results of the PCR-RAPD reactions were processed in a binary system where 1 denoted the presence of a band and 0 the absence of a particular amplification product. The numbers of polymorphic and monomorphic amplification