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Studies on Inter and intra-population variability of *Pongamia pinnata*: a bioenergy legume tree

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ABSTRACT - Pongamia pinnata *is an oil producing tree species with multiple uses and considerable potential as a bioenergy crop. The present investigation has been carried out to assess the extent of genetic structure in a representative set of 111 individuals of* P. pinnata *encompassing seven populations as a prelude for utilization of promising and genetically divergent material in the breeding program. Molecular polymorphism was 67.18% with 10 Inter Simple Sequence Repeats (ISSR) between the individuals indicating modest levels of genetic variation in the P. pinnata germplasm collected. The within population variation based on ISSR polymorphism was 32.34% and polymorphism at the species level was 94.34%. Genetic differentiation between populations (G_{ST}= 0.61) was positively correlated with geographical distance. The data obtained indicated an immediate need for widening the genetic base of* P. pinnata *germplasm for proper characterization and extensive plantations of elite varieties to meet the biodiesel demands.*

Key words: Bioenergy crop, ISSR markers, population diversity.

INTRODUCTION

Pongamia pinnata (Linn.) Pierre is an arboreal legume tree under family Leguminosae and high potential for oil. This tree is a potential for the biodiseal industry (Scott et al. 2007). The oil is also used as a lubricant, water-paint binder, pesticide and in tanning industries (Burkill 1996). Medium size trees are indigenous to the Indian subcontinent and South-East Asia, and have been successfully introduced to humid tropical regions of the world as well as parts of Australia, New Zealand, China and the United States. The mature tree can withstand water logging and slight frost and is highly tolerant to salinity and is common along seashores with its roots in fresh or saltwater (Kuashik et al. 2007). It occurs naturally in lowland forests on limestone and rocky oral outcrops on the coast, along the edges of mangrove forests and along tidal streams and rivers. It is also drought resistant and well adapted to adverse climatic conditions and soil moisture conditions. Its root, bark, leaves, sap, and flower have medicinal properties and have an effect on a wide array of organisms including insects and pests, nematodes and molluses (Srinivasan et al. 2003, Baswa et al. 2001).

As the plant is an efficient and emerging biodiesel crop, it is necessary to establish the evolutionary history, forest fragmentation, genetic processes, and demographic phenomena of diversity and genetic structure in the population. During the evolutionary history, *P. pinnata* has been exposed to long-term geographic isolation, which may have resulted in a decrease in genetic diversity. A low level of genetic variation is problematic for further adaptation to changing environments. The present study of genetic

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diversity in the native range of *P. pinnata* is relevant for the planning of conservation actions to secure the maintenance of the species long-term variability and viability. Better knowledge of the level and distribution of the genetic diversity among populations collected from different regions of Orissa, India enables more realistic conservation and restoration strategies of *P. pinnata*.

MATERIAL AND METHODS

Plant material

A representative set of 111 individuals of *P. pinnata*, across the seven populations from three different eco-geographical regions of Orissa, India were sampled (Table 1). The origin of each population has been shown in Figure 1. Selected populations were heterogeneous for habitat type, population density and degree of exploitation and populations located within the same geographical region did not necessarily share environmental attributes. A neighborhood size of 1.0 Km radius was adopted as the working definition of a population and the population within the same area was separated by a minimum of 5 Km. For ISSR analysis, the number of samples per population varied from 10 to 35 according to population size, the maturity of tree and the availability of the following conditions: leaves were sampled from trees at least 10 m apart, ± 6 m height and with a diameter at breast height ± 80 cm.

DNA Extraction

Genomic DNA was extracted and purified from young leaves using the N-Cetyl-N,N,N-trimethylammonium bromide (CTAB) method as described by Doyle and Doyle (1990) with modifications. The DNA pellet was resuspended in 200 µL to 300 µL of Tris-EDTA buffer. DNA quantification was performed by visualization under UV light, after electrophoresis in 0.8% (w/v) agarose gel. The resuspended DNA was then diluted in sterile distilled water to a 5 ng μL^{-1} concentration for use in amplification reactions.

PCR amplifications

Polymerase chain reactions (PCR) with a single primer were carried out in a final volume of 25 µL containing a 20 ng DNA template, 100 mM of each deoxyribonucleotide triphosphate, 20 ng of primer, 1.5 mM $MgCl_2$, 1x Taq buffer (10 mM Tris-HCl [pH-9.0], 50 mM KCl, 0.001% gelatin), and 0.5 U Taq DNA polymerase (M/S Genei, Bangalore, India). Amplification was performed in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) programmed for a preliminary 2 min denaturation step at 94 °C, followed by 40 cycles of denaturation at 94 °C for 20 sec, annealing at the required temperature for 30 sec and an extension at 72 °C for 1 min, finally at 72 °C for 10 min amplification. Amplification products were separated alongside a molecular weight marker (1.0 Kb plus ladder, M/S Bangalore Genei, Bangalore, India) by 1.5 % agarose gel electrophoresis in 1x TAE (Tris Acetate EDTA) buffer stained with 0.5 ug mL^{-1} ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System (Gel Doc. 2000, BioRad, California, USA) and the amplification product sizes were evaluated using the software Quantity one (BioRad, California, USA).

Table 1. Eco-geographic data of the location from where *P. pinnata* populations collected

Location	Regions	Code	GPS reading		Altitude	Climatic parameter		N
			Latitude (°N)	Longitude (°W)	(m)	Tm (°C)	Rn (cm)	
Bargaon	Shambalpur	PSB	22°07'	84°03'	450	27.0-32.0	60-100	25
Kalimati	Shambalpur	PSK	21°30'	83°59'	450	27.0-32.0	60-100	15
Ghatikia 1st population	Bhubaneswar	PBG-I	20°08'	85°58'	220	25.0-29.5	200-600	50
Ghatikia 2nd population	Bhubaneswar	PBG-II	20°08'	85°58'	220	25.0-29.5	200-600	26
Kosola	Anugul	PAK	21°02'	85°26'	220	25.0-39.5	200-400	65
Dera	Anugul	PAD	21°16'	85°29'	220	25.0-39.5	200-400	20
Katada	Anugul	PANK	21°01'	85°27'	220	25.0-39.5	200-400	25

Tm- Annual temperature; Rn- Annual rainfall; N- Number of individuals collected

Figure 1. Location of different plots from where *Pongamia pinnata* were collected

Data analysis

Polymorphic ISSR bands were scored as present (1) or absent (0). Popgene, v. 1.31 (Yeh et al. 1999) was used to calculate the genetic diversity for each population, including the mean number of alleles per locus (A) and the percentage of polymorphic loci (*P*). The level of gene flow (*Nm*) was measured using Nei's (1973) gene diversity statistics. The Shannon information measure (H_o) was calculated at two levels: the average diversity within populations (H_{pop}), and the total diversity within species (H_{sp}). The proportion of diversity between populations $D = (H_{sp} - H_{pop})/H_{sp}$ was estimated. The binomial matrix was used to calculate the level of polymorphism for each population. Within-population diversity values were calculated using Nei's unbiased diversity statistic, averaging over individual ISSR products. A UPGMA dendrogram showing the relationships between populations based on Nei's genetic distance (Nei 1978) was also constructed using software Popgene.

RESULTS AND DISCUSSION

The effectiveness of the tree improvement program depends upon the nature and magnitude of existing genetic variability and also on the degree of traits transmission or heritability (Zobel and Talbert 1984) because genetic variation is the fundamental requirement for maintenance and long-term stability of forest ecosystems. The results showed that out of the twenty-seven ISSR primers evaluated with two individuals across the seven populations, seventeen primers either gave a smear on the gel or resulted in faint or irreproducible bands. Ten primers were selected owing to their reliability and good-quality fingerprints. Ninety polymorphic bands were obtained out of a total of 136 amplified bands, revealing 67.14% polymorphism for the ten ISSR primers analyzed. The percentage of polymorphism over all populations varied from 37.5% (IG-06) to 86.6% (IG-14) between primers (Table 2). The primer IG-11 amplified a maximum of the highest bands (19) where as primer IG-06 was the least being 08 amplicons only. ISSR fragments ranged from 125 to 2500 bp (Table 3a). A polymorphism of 94.34% was observed at the species level either between or within populations, across our entire sample. The percentage of polymorphic loci (P) varied greatly between the three regions and seven populations. For a single population, it ranged from 19.81 % (PANK) to 44.30% (PBG-I), with an average of 32.34% across the seven populations. A moderate level of genetic diversity within populations was observed, and these standard measures of genetic diversity varied in all populations. Among the seven populations, two populations from the Bhubaneswar region PBG-I (P: 44.34% and H_o: 0.13) and PBG-II (P: 38.68% and H_o: 0.11) exhibited the greatest level of

Table 2. ISSR primers used for molecular analysis

Primer	Primer sequence	Total No of bands	No of monomorphic bands	No of polymorphic bands	% polymorphism
IG-09	3'-$(AG)^8$C-5'	12	05	07	58.33
IG-10	3'-$(AC)^8$T-5'	14	06	08	57.14
IG-11	3'-$(AC)^8$G-5'	19	08	11	57.89
IG-12	3'-$(GA)^8$C-5'	12	05	07	58.33
IG-14	3'-$(GA)^8$T-5'	15	02	13	86.66
IG-13	3'-$(GA)^8$A-5'	12	04	08	60.00
IG-23	3'-$(GACAC)_2$-5'	17	03	14	82.35
IG-03	5'-GAGGGTGGAGGATCT-3'	17	04	13	76.47
IG-06	5'-GACAGATAGACAGATA-3'	08	05	03	37.50
IG-15	3'-$(CT)^8$G-5'	10	04	06	60.00

variability, while the population PANK from the Anugul region exhibited the lowest (P: 19.81% and H_o: 0.06). At the population level, the average values of A_e, H_o and P for all the populations were 1.18, 0.10 and 32.34%. At the species level A_e, H_o and P were 1.48, 0.28, and 94.34%, respectively. The Shannon indexes (Io) ranged from 0.10 to 0.20, with an average of 0.16 at the population level (H_{pop}) and 0.43 at the species level (H_{sp}). The Shannon's diversity index (D) was 62.79%, suggesting that a high genetic differentiation existed between populations. The coefficient of genetic differentiation between populations was 0.61 (Table 4) as estimated by the partitioning of the total gene diversity (G_{ST}). The level of gene flow (based on G_{ST}) estimated was very low (Nm = 0.30). Kuashik et al. (2007) reported that the distribution of genetic variability is limited in *P. pinnata* on the basis of their morphological characteristics such as pod and seed traits. The proportion of polymorphic loci amplified across the species level in *P. pinnata* was 94.34%, which is similar to figures reported for other tropical tree legumes such as *Caesalpinia echinata* Lam. (Cardoso et al. 1998) and to those reported in *Populus tremuloides* (Yeh et al. 1995) using random amplified polymorphic DNA.

The genetic relationships between populations were analyzed on the basis of Nei's genetic distance (Nei 1978). The data ranged from 0.008 for the most closely related populations (PAD & PAK) to 0.418 for the most divergent population (PANK and PBG-II) (Table 5). In general, there was a positive correlation between geographical and genetic distance that was confirmed by the Mantel test ($r = 0.813$, $p < 0.001$). The UPGMA phylogenetic tree (Figure 2) based on pair wise genetic distances further highlighted the correlation between genetic and geographical distances. Populations were grouped into three distinct clusters owing to their regional origin. The three populations from the Anugul region (PAK, PAD and PANK) formed a distinct group, as did the two populations from the Sambalpur region (PSB and PSK) and the two populations from the Bhubaneswar region (PBG-I and PBG-II) groups in the phylogram. The results of the analysis, therefore, showed a distinct geographical partitioning of genetic variance, and the estimates of $Nm = 0.30$ revealed a low level of migration between the isolated locations, which resulted in considerable geographical differentiation. Similar results concerning genetic structure of *P. pinnata* populations were obtained using Shannon's diversity index (D = 62.79 %). Moderate low diversity and high population partitioning in tree species have previously been attributed to a number of factors, including the adaptation of genetic systems in small populations, recent fragmentation of continuous genetic systems (human activity), and limited gene flow due to the combination of wind pollination and a high inbreeding rate (Maguire and Sedgley 1997). Nevertheless, at the species level the results are consistent with data from other tree species, in which high genetic variations have been related to life history and ecological characteristics such as a wide geographical range, primarily out crossing and animal-

Table 4. Genetic population structure and estimate of gene flow within the populations of *Poingamia pinnata* collected from different silviculture plots

Markers	H_T	H_S	G_{ST}	Nm
136	0.29 (0.02)	0.11 (0.008)	0.61	0.30

H_T: Total variability; H_S: Variability within population; G_{ST}: inter population differentiation; Nm: gene flow within population

Table 3. Mean genetic data of populations collected from different silviculture plots

Population	A_o	A_e	H_o	I_o	P	% P
PSB	1.32(0.46)	1.21(0.36)	0.11(0.19)	0.17(0.27)	34	32.08
PSK	1.28(0.45)	1.18(0.33)	0.10(0.18)	0.15(0.26)	30	28.30
PBG-I	1.44(0.49)	1.21(0.31)	0.13(0.17)	0.20(0.26)	47	44.34
PBG-II	1.38(0.48)	1.17(0.29)	0.11(0.16)	0.17(0.24)	41	38.68
PAK	1.33(0.47)	1.22(0.36)	0.12(0.19)	0.18(0.27)	35	33.02
PAD	1.30(0.46)	1.21(0.35)	0.11(0.19)	0.17(0.27)	32	30.19
PANK	1.19(0.40)	1.11(0.27)	0.06(0.15)	0.10(0.22)	21	19.81
Mean	1.32(0.45)	1.18(0.32)	0.10(0.17)	0.16(0.25)	34	32.34
At species level	1.94(0.23)	1.48(0.35)	0.28(0.16)	0.43(0.21)	100	94.34

A_o: Observed Number of Alleles; A_e: Effective Number of Alleles; H_o: Nei's Gene Diversity; Io: Shannon's Information Index; P: Number of polymorphic bands; % P: Percentage polymorphism

seed dispersal mechanisms (Hamrick and Loveless 1989). Genetic diversity is essential to the long-term survival of tree species to avoid the risk of extinction. The loss of genetic variation is thought to decrease both the short-term and the long-term adaptability of populations in variable and changing environments.

Table 5. Nei's measures of genetic identity (above diagonal) and genetic distance (below diagonal) between the populations of *P. pinnata* by ISSR markers

Populations	PSB	PSK	PBG-I	PBG-II	PAK	PAD	PANK
PSB	-	0.973	0.741	0.730	0.714	0.704	0.689
PSK	0.027	-	0.727	0.715	0.710	0.704	0.689
PBG-I	0.299	0.318	-	0.973	0.690	0.678	0.661
PBG-II	0.314	0.334	0.027	-	0.687	0.672	0.658
PAK	0.335	0.342	0.370	0.375	-	0.991	0.954
PAD	0.349	0.350	0.388	0.396	0.008	-	0.945
PANK	0.371	0.371	0.413	0.418	0.046	0.055	-

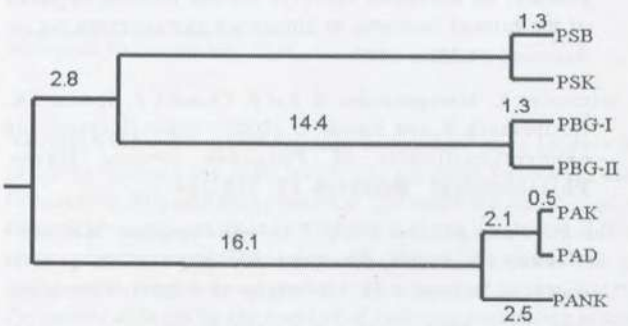

Figure 2. Dendrogram showing the cluster analysis between the seven populations of *P. pinnata* using ISSR markers. Number in the internode regions indicates the genetic distance

CONCLUSION

The study is to provide information on the extent of genetic variability available in the *Pongamia* germplasm that is essential for the conservation and breeding program. The maintenance of high genetic diversity in *P. pinnata* is one of the most important issues for sustainable *P. pinnata* forest in the future. It will help to provide a genetic input into the management of *P. pinnata* for conservation purposes and will also focus on a wider range of populations to widen the genetic base and for further genetic improvement of this crop.

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Estudo da variabilidade inter e intra-populacional de *Pongamia pinnata*: uma leguminosa arbustiva para a bioenergia

RESUMO - Pongamia pinnata *é uma espécie arbórea de múltiplos usos, produtora de óleo e com grande potencial bioenergético. Esse estudo foi conduzido para verificar a existência de estrutura genética em um grupo representativo com 111 indivíduos de* P. pinnata *de sete populações distintas para utilização posterior em ensaios de material promissor e geneticamente divergente no programa de melhoramento. O polimorfismo molecular entre os indivíduos foi de 67,18%, utilizando 10 marcadores ISSR (Inter Simple Sequence Repeats), indicando modestos níveis de variação genética no germoplasma de* P. pinnata. *A variação dentro das populações com base no polimorfismo ISSR foi de 32,34% e o polimorfismo, ao nível de espécie, foi 94,34%. A diferenciação genética entre as populações (G_{ST} = 0,61) foi positivamente correlacionada com a distância geográfica. Os dados obtidos indicam necessidade imediata de ampliação da base genética do germoplasma de* P. pinnata *para uma caracterização adequada e para implantar extensas plantações com variedades selecionadas para atender à demanda na produção de biodiesel.*

Palavras-chave: Produção de bioenergia, marcadores ISSR, diversidade populacional.

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