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Molecular Characterization of *Pongamia pinnata* (L.) Pierre Genotypes in Respect to Seed Oil Using Microsatellite Markers

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Twenty-four genotypes selected from different agroclimatic zones of Jharkhand (India) were assessed for seed oil content that varied from 21.86% to 41.86% with 14 genotypes recording above average 32.11 % for the trait which indicates towards efficiency of selection processes. Several genotypes in 'Central and Western Plateau' agroclimatic zone of Jharkhand displayed a good potential for high oil content. The employed 23 polymorphic microsatellite loci exhibited three to twenty one alleles per locus with an average of 12 while total 270 alleles were detected. Two primer set PpSSR21 and PpSSR27 showed 100% polymorphism among the genotypes. The high oil yielding plant K_{19} showed different band pattern with the locus PpSSR04. From the Nei's analysis it was found that maximum diversity exists between the full sib genotypes K_{10} and K_2. Thus, the genotypes (K_2 and K_{10}) which are more diverse could be used further in improvement programme. Overall, the genotypes included in the study showed a correlation with their geographical origins such that genotypes from the same region tend to have higher genetic

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similarity as compared to those from different regions. However, in UPGMA based Nei's analysis, some genotypes were found not to be grouped based on geographical origins possibly due to the exchange of germplasm over time between farmers across the regions. Both molecular and oil content (biochemical) markers appeared useful in analyzing the extent of genetic diversity in *P. pinnata.* The result of these analyses will help to better understand the genetic diversity and relationship among populations constituting a set of useful background information that can be used as a basis for future breeding strategy and improvement of the species.

Keywords: Amplicons; biofuel; genetic diversity; SSR markers; polymorphism.

1. INTRODUCTION

With declining reserves of fossil fuels, biofuels can make a substantial contribution to the future energy demands of the domestic and commercial economies. Among many TBOs (Tree Bourne Oilseed species) of India, which can yield oil as a source of energy in the form of biodiesel, Karanz [*Pongamia pinnata* (L) Pierre] with 32-42% oil content has been found to be the most suitable species due to its various favorable attributes like high oil recovery, quality of oil containing high amount of polyunsaturated fatty acids which are an important source of biodiesel and subsequent processing of biodiesel [1-4]. It is a leguminous deciduous tree, commonly known as Indian Beech, Pongam, Honge and Karanj, grows about 15-25 m tall and is well-adapted to semi-arid and humid zones.

P. pinnata is indigenous to the Indian subcontinent and has been successfully introduced to humid tropical regions of the world as well as parts of Australia, New Zealand, China and the United States [5]. The species easily growing on unproductive land and adaptable to wide agro-climatic conditions, can prove itself to be a promising alternative to the energy requirement in the future especially in a country like India which produces only 22% of its diesel requirement and 78% is imported at a huge cost in foreign currency. However, central to the domestication and sustained use of *P. pinnata*, is an understanding of its molecular genetics including aspects of gene content and structural organization in relation to other plant genomes [6].

In meeting future demands for bio-diesel establishment of extensive commercial-scale *P. pinnata* plantations will be beneficial necessitating identification and selection of *P. pinnata* plants that have genetically linked with optimal production of biofuels, such as high seed oil content. The effectiveness of tree improvement targeting this will depend upon the nature and magnitude of existing genetic variability in the species for which molecular approach is more efficient as it directly quantifies genetic variability at DNA level. Molecular markers have provided a powerful tool for breeders to search for new sources of variation and to investigate genetic factors controlling quantitatively inherited characters. Various PCR based molecular markers have been employed in *Pongamia* for identification, phylogenetic analysis, population studies and genetic linkage mapping [7-14]. Simple sequence repeats (SSR) or microsatellite markers have emerged as the markers of choice due to several desirable attributes including their abundance, multiallelic and co-dominant nature, high level of reproducibility, cross-species transferability etc. for such studies.

Keeping these facts in mind present investigation involving microsatellite markers was conducted to study the existing genetic diversity and variation in different genotypes of *P. pinnata* collected from representative parts of Jharkhand and to document their potentiality for exploitation of oil yielding property as a scope for their incorporation in further breeding program.

2. MATERIALS AND METHODS

2.1 Plant Material

Germplasm of twenty four genotypes of *P. pinnata* collected from different region of Jharkhand and maintained at Institute of Forest Productivity, Ranchi, Jharkhand (India) as clonal (grafted plants) were utilized (Table 1). Three individual of each of 24 genotype (clonal) were selected for the study.

Table 1. Details of *Pongamia pinnata* genotypes used for genetic diversity analysis

Sl. No.	Code	Forest Division	Latitude	Longitude	Altitude (m)	Agroclimatic zone
1	K_{13}	Hazaribag North	24°27'10″N	85°31'42″E	370	JH-1
2	K_{25}	Giridih	24°17'11″N	86°21'55″E	390	Central and North
3	K_{12}	Hazaribag West	23°54'19″N	85°19'04″E	610	eastern Plateau
4	K_{14}	Koderma	24°27'21″N	85°46'12″E	380	Zone
5	K_{11}	Chatra South	23°54'19″N	85°19'04″E	640	
6	K_{19}	Ranchi	23°33'02″N	84°54'43″E	790	JH-2 Central and
7	K_{27}	Ranchi East	23°17'07″N	85°10'35″E	810	Western Plateau
8	K_{09}	Lohardaga	23°33'02″N	84°54'43″E	640	Zone
9	K_{15}	Ranchi East	23°27'45″N	85°28'39″E	630	
10	K_{23}	Khunti	23°03'05″N	85°13'40″E	700	
11	K_{10}	Garhwa South	23°55'30″N	83°46'11″E	410	
12	K_{07}	Simdega	22°35'47″N	84°40'49″E	370	
13	K_{03}	Gumla	23°07'02″N	84°33'21″E	520	
14	K_{04}	Lohardaga	23°26'17″N	84°38'36″E	590	
15	K_{26}	Ranchi	23°28'40″N	85°10'17″E	790	
16	K_{18}	Ranchi East	23°09'04″N	85°28'40″E	500	
17	K_{02}	Ranchi East	23°28'36″N	85°01'06″E	610	
18	K_{24}	Ranchi East	23°33'55″N	85°05'05″E	650	
19	K_{06}	Lohardaga	23°21'06″N	84°39'16″E	570	
20	K_{20}	Gumla	23°08'22″N	84°46'47″E	800	
21	K_{21}	Gumla	22°52'39″N	84°53'36″E	500	
22	K_{22}	Porahat	22°52'35″N	85°18'15″E	690	JH-3
23	K_{17}	Dhalbum	22°27'10″N	86°37'09″E	350	South Eastern
24	K_{16}	Saraikela	22°51'42″N	85°56'55″E	390	Plateau Zone

2.2 Biochemical Analysis

Seed of different genotypes of *P. pinnata* was analyzed for total oil content as per Sadasivam and Manickam [15].The data obtained were subjected to statistical analysis, employing analysis of variance (ANOVA), 'F'-test for significance at $P \leq 0.05$ and computing LSD values to separate means in different statistical groups using statistical software IBM SPSS version 18.

2.3 DNA Extraction and SSR Analysis

Total DNA was extracted from fresh leaves by the cetyl tri-methyl ammonium bromide (CTAB) method with slight modification [16,17]. The quality and concentration of extracted DNA were estimated by using a UV spectrophotometer (Biophotometer plus, Genetics) and running an aliquot on 0.8% agarose (Sigma-A9539) gels. After visualization the DNA was diluted if required.

2.4 Primer Designing

Specific primers were designed for the flanking regions of the SSRs using the software WEBSAT (wsmartins.net/websat/). The sequences would be downloaded from public databases (nucleotide sequences at the NCBI databases (http://www.ncbi.nlm.nih.gov)). The designed primers were of 19–25 bp length, annealing temperature was 50–60°C, and product sizes were 100 to 400 bp (Table 2). The criteria adopted to reduce the amplification of nonspecific bands in PCR reactions included a minimum primer annealing temperature (Tm) of 57°C, a maximum difference of 1°C in Tm between the two primers of an SSR locus and a G+C content ranging from 40 to 80°C. The primers were synthesized by SIGMA-ALDRICH (USA). Total twenty nine primer pairs (mono, di and trinucleotide base) designed were tested for DNA amplification on twenty four *P. pinnata* genotypes.

2.5 Standardization of Annealing Temperature

The best annealing temperatures for SSR primers pair were standardized. For each primer a gradient PCR was performed. Six primer pair did not show any amplification at different annealing temperature. So, only twenty three microsatellite Primer pair was taken for this study (Table 3).

Table 2. Different primers set designed and employed for the study

Sl. No.	Primer code (Locus)	Sequence	Sl. No.	Primer code (Locus)	Sequence
1	PpSSR02 F	GCATCACCAACAAAAGCATCTA	13	PpSSR 14 F	GAGACCGCATACAACCTTCAAT
	PpSSR02 R	AACCCACTTTCTTCTCCCTTTC		PpSSR 14 R	CCTTTTGTTGGGAAGAAGATGT
2	PpSSR03 F	CACGAAAGGAGGAAGGTATGTC	14	PpSSR 15 F	TGTGAATGTGAGTGATGTTGGA
	PpSSR03 R	CACTACCCAGCAAATAGCAGATT		PpSSR 15 R	CTTTGTGTTGAGTGTGTGTGGA
3	PpSSR04 F	CTCGTTGAGACCATGTTAGAAA	15	PpSSR 16 F	TCGAAGTTGTATGTAACGGTGG
	PpSSR04 R	CACACACACACACACACAATCT		PpSSR 16 R	TTTGACTCCTCCTCTTCTCTCG
4	PpSSR05 F	GTGGAGTCTTGGGATGTGTGTA	16	PpSSR 17 F	CTTACCTTTAGGGATAGAATTTGCC
	PpSSR05 R	GACTGAGCTGTCAACCTTGGAT		PpSSR 17 R	GATCCTTGTTGTTCTTGCCATT
5	PpSSR06 F	GATGATGAGGATACTGCCATGA	17	PpSSR 18 F	GCATGAGCAATAAGGTGGC
	PpSSR06 R	AACATAACAATACGTGAGCCCC		PpSSR 18 R	TCCCCTAAATCAACTCTTCATAGG
6	PpSSR07 F	TGGTATCTTTGGCATTGTGTTG	18	PpSSR 19 F	TGTTTTGTCCACTGTCAACCAT
	PpSSR07 R	CAACCACTTATCCTCAAGGGAG		PpSSR 19 R	CATGATTCGGTTTCTGCAAGT
7	PpSSR08 F	GGAATCTCGACACCTAAACTGG	19	PpSSR 21 F	ATTGGTTGGTTGGTGCTAGAGT
	PpSSR08 R	TCAGACCAATTCACCACGATAC		PpSSR 21 R	CCTTCCTCTTTTCTCCCTCATT
8	PpSSR09 F	CCTTTCATTCCCTTGTTTCGT	20	PpSSR 22 F	CTTATCGGTGCAAGACAACAAC
	PpSSR09 R	ATTCCCAGTAGTAAGTCGTCGG		PpSSR 22 R	TCTCTCTCTCTCTCGCTTTGCT
9	PpSSR10 F	GGAATCTCGACACCTAAACTGG	21	PpSSR 27 F	AGACTCACCACTCAAATGCTTG
	PpSSR10 R	TTCAGACCAATCACCACGATAC		PpSSR 27 R	CCTCATCTTTCCTTCCCTCTTT
10	PpSSR11 F	GAGAGAAAGTGTGTAACGAGAGAGG	22	PpSSR 28 F	ATGGTTGGTTGGTGCTAGAGTT
	PpSSR11 R	ATCCCCTCCAATTTTCGTAAG		PpSSR 28 R	CCTTCCTCTTTTCTCCCTCATT
11	PpSSR 12 F	GAGAGAAAGTGTGTAACGAGAGAGG	23	PpSSR 29 F	CTTATCGGTGCAAGACAACAAC
	PpSSR 12 R	CCCCTTCAAATTCATCATCATC		PpSSR 29 R	TCTAACCCTCTCTCTCCCTCAA
12	PpSSR 13 F	AGGATGTGGATGTGTCAAAGAG			
	PpSSR 13 R	CTGTCCCAATCAATTTCACCTA			

F=Forward R=Reverse

Table 3. Particulars of simple sequence repeat primers used

Sl. No.	Primer code (Locus)	Length of the primer	GC%	Repeat Motif	Amplicons size generated by the software (in bp)
1	PpSSR02 (F,R)	22,22	41,45	(GA)10	110
2	PpSSR03 (F,R)	22,23	50,43	(TG)13	247
3	PpSSR04 (F,R)	22,22	41,45	(A)10	333
4	PpSSR05 (F,R)	22,22	50,50	(GAA)15	196
5	PpSSR06 (F,R)	22,22	45,45	(GAG)6	162
6	PpSSR07 (F,R)	22,22	41,50	(GT)9	147
7	PpSSR08 (F,R)	22,22	50,45	(TG)12	273
8	PpSSR09 (F,R)	21,22	43,50	(A)10	165
9	PpSSR10 (F,R)	22,22	50,45	(TG)12	191
10	PpSSR11 (F,R)	25,21	48,42.8	(GAT)6	235
11	PpSSR12 (F,R)	25,22	48,40.9	(GA)15	110
12	PpSSR13 (F,R)	22,22	45.4,40.9	(AGA)6	247
13	PpSSR14 (F,R)	22,22	40.9,45.4	(CA)21	333
14	PpSSR15 (F,R)	22,22	45.4,50	(AG)8	196
15	PpSSR16 (F,R)	22,22	40,40.9	(AG)9	162
16	PpSSR17 (F,R)	25,22	52.6,41.6	(AC)9	147
17	PpSSR18 (F,R)	19,24	40.9,42.8	(ATG)6	273
18	PpSSR19 (F,R)	22,21	45.4,45.4	(AC)10	165
19	PpSSR21 (F,R)	22,22	45.4,50	(AG)10	304
20	PpSSR22 (F,R)	22,22	45.4,45.4	(GA)19	240
21	PpSSR27 (F,R)	22,22	45.4,45.4	(GAA)6	151
22	PpSSR28 (F,R)	22,22	45.4,45.4	(AG)9	299
23	PpSSR29 (F,R)	22,22	45.4,50	(GA)8	378

2.6 PCR Conditions for SSR Primers

Each PCR reaction was carried out in a 20 µl reaction volume containing 10 pmoles each of forward and reverse primer, 1 Unit Red Taq DNA polymerase (Banglore GeNei), 10X Taq buffer A, 200 µM of deoxyribonucleotides and 50 ng of template genomic DNA was added. The temperature profile used for PCR amplification comprised denaturation at 94°C for 4 min, followed by 35 cycles of 92°C for 30 sec, the annealing temperature for 30 sec , 72°C for 1 min and a final extension was given at 72°C for 5min. The annealing temperature was adjusted basing on the specific requirements of each primer combination. The amplicons obtained after PCR was run on 3.5% agarose gel electrophoresis (wide range agarose, Sigma A 7431). Appropriate ladders were also run with the samples. The samples were electrophoresed at constant voltage of 180 Volts (5V/cm) for 3 hours. The gel was then visualized under UV light on a transilluminator and documentation of the samples was done on Gel Documentation System (Syngene).

The amplified products were arranged according to their molecular weight and according to that a particular genotype was assigned to each variety. Each value represents a particular allele. The genotype of all the individuals were fed in the software and on its basis genetic similarity and variation was analyzed. The genotype was assigned a null allele for a microsatellite locus whenever an amplification product could not be detected for a particular genotype-marker combination. The calculations were performed by using Popgene software [18]. Percentage of all loci that was polymorphic regardless of allele frequencies was determined using the software.

Nei's analysis of genetic distance and identity was used to study the genetic distance between the populations [19]. A dendrogram were created with UPGMA (unweighted pair group method with arithmetic averaging) based on Nei's genetic distance. Ewens-Watterson test are used to test the neutrality of the locus. The observed F value is compared with the lower and upper 95% confidence interval. If observed F value is within this confidence interval, the locus is neutral otherwise it is not. The distribution of private (unique) alleles was assessed as a mean to describe population distinctiveness [20] and levels of genetic interrogation between populations [21]. Private alleles were assessed manually from the converted data file for allelic frequency with a software Convert1.31 [22].

3. RESULTS AND DISCUSSION

3.1 Biochemical Characteristics

Seed from different regions contain a lot of variations in morphological and physiological characteristics which could be genetic in nature as a result of adaptation to diverse environmental condition prevailing throughout their distributional range [23]. In the present study also significant variation among different genotypes of *P. pinnata* in total oil content was recorded which provides an opportunity for genetic improvement and breeding in the species.

The highest seed oil content (41.87%) was found in the K_{19} followed by K_{27} (39.33%) and K_{22} (38.80%). Average total oil content was 32.11% while seeds of 14 genotypes recorded above average for the trait (Fig.1).The average acid value (mg KOH/g) was 11.07 while the average Iodine number was 76.09. The identified promising candidates such as K_{19}, K_{27}, K_{22}, K_{09} and K_{15} were can be used for clonal development and utilization in large scale plantation drives of *P. pinnata*. Based on the average oil content at agroclimatic zone, the order of *Pongamia* genotypes from high to low was fifth agroclimatic zone (Central and Western plateau, JH-2) in India< fourth agroclimatic zone in India (Central and North Eastern Plateau , JH-1) < sixth agroclimatic zone in India (South Eastern Plateau, JH-3). Some mixing however was also observed.

Central and Western plateau (JH-2) accounts for 33% natural forests of Jharkhand and characterized by late arrival, early cessation and uneven distribution of monsoon rains; and poor water retention capacity of soil. These conditions might have an effect on enhanced seed oil in the genotypes of the region. However, from overall evaluation of seed oil content, it appears that individual genotypic factors supersede over environmental factors in determining oil yield.

3.2 Molecular characterization through SSR markers

The genetic marker can be used to select wild candidate plus trees collected from different agro-climatic regions of tree improvement programs. Various molecular approaches have been employed for genetic characterization of *P.*

pinnata such as RAPD [7,8,24,25], AFLP [26], ISSR [27,28], TE-AFLP [8,10,26,28] and SSR[28]. However there are no reports available on the variability in total seed oil content of *P. pinnata* with the help of SSR markers.

For the use of SSRs as markers, it is necessary to design the primers. The SSRs commonly used for marker development are those belonging to di-, tri- and tetra-nucleotides. The mononucleotides are useful for population genetic analyses of chloroplast genomes and can also be useful in filling gaps in linkage maps created by di-, tri-, and tetra-nucleotide repeats but, at the same time they cause difficulties in accurate sizing of polymorphisms.

In the present study, species specific SSR primers were designed with the help of WEBSAT software. Development of SSRs by data mining from sequence data is a relatively easy and cost-saving strategy for any organisms with enough DNA data. This is a very good example from data to knowledge and from knowledge to basic and applied studies for biology, production, conservation and management of many organisms. The primer sets were selected on the basis of the product length of the amplicons which ranged from 110-400bp. By employing high resolution agarose gel electrophoresis we were successful to clearly separate alleles for the twenty three microsatellite markers studied.

3.3 Amplification Profiles and Allelic Variants

The twenty three polymorphic microsatellite loci identified in this study showed a clear and single or multiple peak for each allele, while remaining six markers showed no clear band in accessions. The amplified products were in agreement with the expected size from sequence data for all 23 microsatellites analyzed except one SSR marker. The distribution of alleles for a single and multiple SSR loci across all genotypes is shown in Figs. 2-3. A total of 270 alleles were detected by the twenty three markers. The number of alleles per locus varied from 3 to 21. The average number of alleles per locus was 12, indicating a greater magnitude of diversity among the plant materials include in this investigation (Table 4).

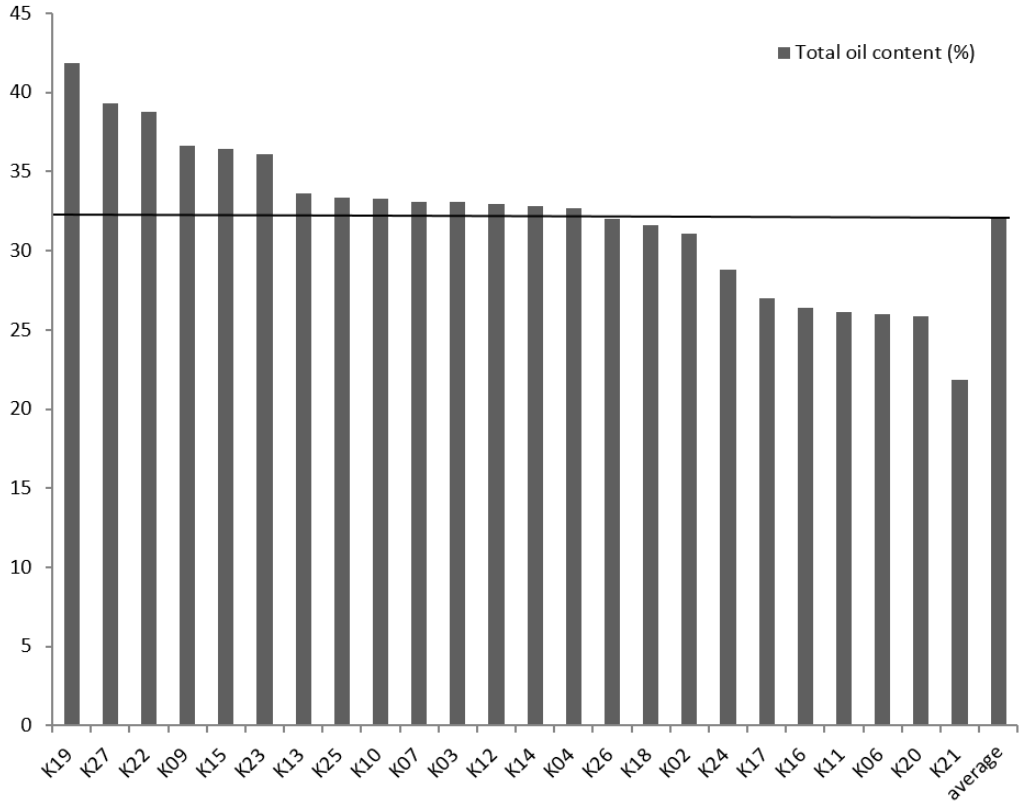

Fig. 1. Total oil content (%) in 24 genotypes of *Pongamia pinnata* recorded above average for the trait

By combined analysis of 11 polymorphic loci (more than 70%) it was possible to distinguish almost all genotypes. However, the complete identification of 24 genotypes implied the study of few additional loci. For the twenty three loci studied we detected 270 different alleles, PpSSR04 and PpSSR09 being the most informative loci for genetic identification because number of alleles were present in single genotypes. So specific allele size was used for particular types of genotypes.

The study also revealed that primers PpSSR12 and PpSSR13 generated a maximum of 21 and 20 bands, while primers PpSSR19, PpSSR27, PpSSR02 and PpSSR15 had a minimum of 3, 5 and 6 bands. Many studies have also reported significant differences in allelic diversity among various microsatellite loci [29,30].

The markers employed in the study showed a high level of polymorphism, ranging from 50-100% (as many as 20 markers). AFLP detected higher levels of genetic diversity (100%) in natural populations of *P. pinnata*, whereas RAPD and ISSR showed lesser genetic diversity (approx. 10%) [8]. The frequency of genotypes revealing null alleles only contained in single primer set PpSSR19. Two primer set PpSSR21 and PpSSR27 showe 00% polymorphism among the genotypes.

The high oil yielding plant K_{19} showed different band pattern with the locus PpSSR04. The amplicon size expected with this primer set was 308bp. The range of the amplicons obtained were 293-322bp. The unique product length 293bp was obtained which is distinct in the genotypes (Fig. 4). So this locus may be used as a marker for high oil yield genotype detection. However, for its documentation, it is necessary to sequence this locus in whole genotype and then differentiate them on the basis of base sequence and further development of SNP.

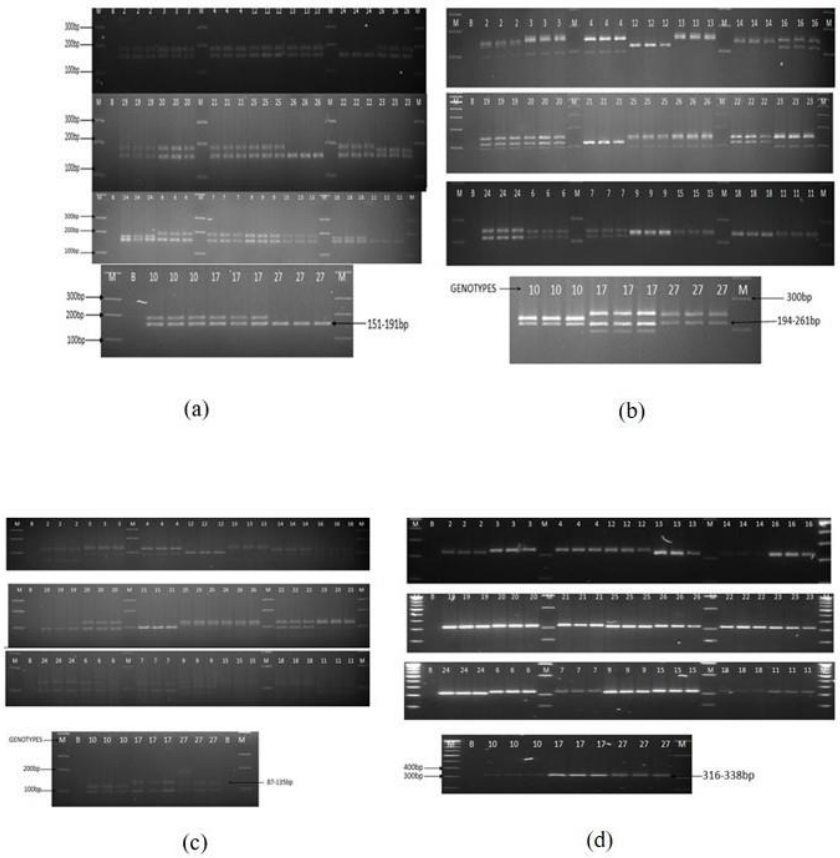

Fig. 2. (a, b, c and d). Gel picture showing the amplicons generated by SSR primer PpSSR5, PpSSR11, PpSSR12 and PpSSR14. Numbers in the top correspond to the *Pongamia* genotypes given in Table 1. M= Molecular markers (100bp ladder, Low range DNA ruler)

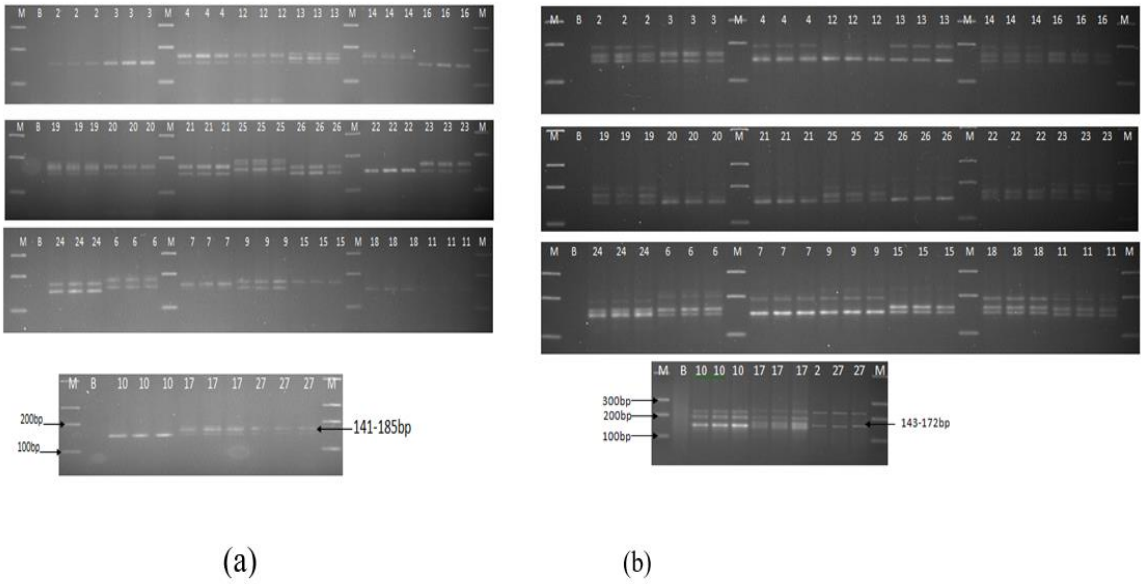

Fig. 3. (a, b). Gel picture showing the amplicons generated by SSR primer PpSSR16 and PpSSR17. Numbers in the top correspond to the *Pongamia* genotypes given in Table 1. M= Molecular markers (100bp ladder, Low range DNA ruler)

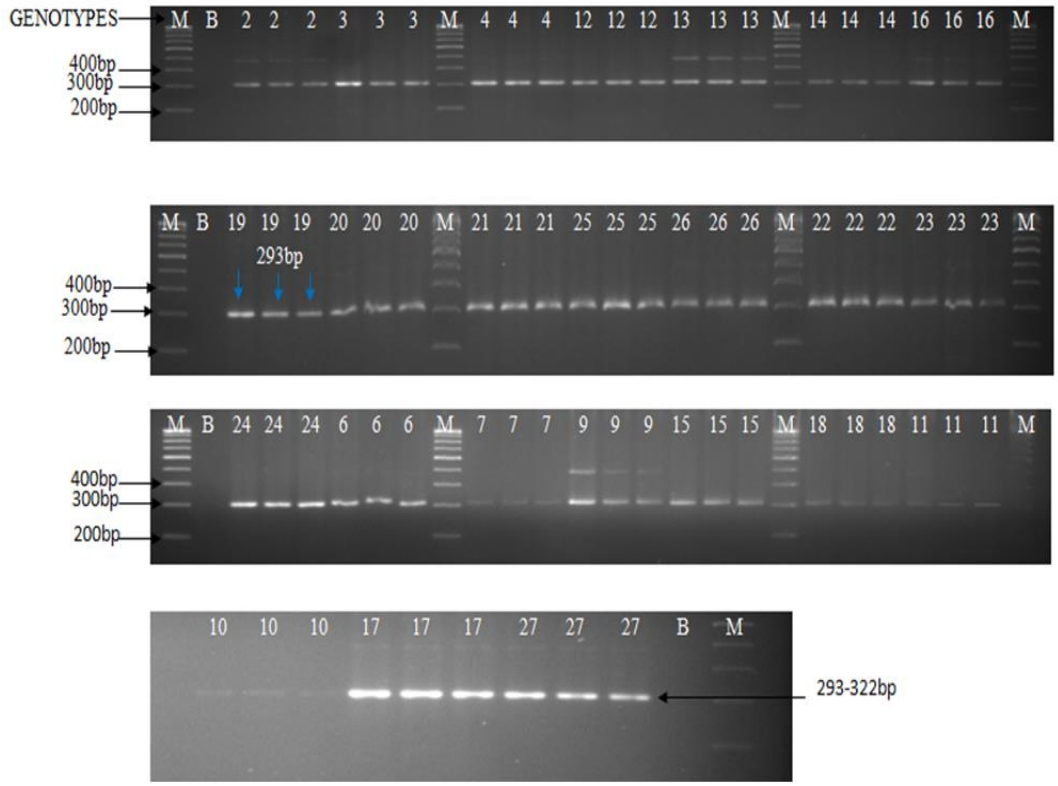

Fig. 4. Different band pattern obtained by K19 at locus PpSSR04

Table 4. Polymorphism shown by microsatellite markers among 24 genotypes of *P. pinnata*

Sl. No.	Locus	Type of repeat (nucleotides)	Product length in WEBSAT software	Amplified product size range in gel (Full sib)	Range of product length	Total number of alleles(Full sib)	Number of Polymorphic alleles	Percentage of polymorph-ism
1	PpSSR02	Di	146	143-154	11	6	4	67
2	PpSSR03	Di	304	296-309	13	7	5	71
3	PpSSR04	Mono	308	293-322	29	16	5	31
4	PpSSR05	Tri	183	151-191	40	9	6	67
5	PpSSR06	Tri	330	295-335	40	11	10	91
6	PpSSR07	Di	221	214-234	20	9	8	89
7	PpSSR08	Di	307	297-330	33	11	8	73
8	PpSSR09	Mono	400	386-416	30	17	5	29
9	PpSSR10	Di	307	301-318	17	8	6	75
10	PpSSR11	Tri	235	194-261	67	17	12	71
11	PpSSR12	Di	110	87-135	48	21	12	57
12	PpSSR13	Tri	247	241-305	64	20	16	80
13	PpSSR14	Di	333	316-338	22	10	7	70
14	PpSSR15	Di	196	190-203	13	6	3	50
15	PpSSR16	Di	162	141-185	44	17	10	59
16	PpSSR17	Di	147	143-172	29	15	11	73
17	PpSSR18	Tri	273	243-284	41	12	11	92
18	PpSSR19	Di	165	166-170	4	3	1	33
19	PpSSR 21	Di	304	294-309	15	7	7	100
20	PpSSR 22	Di	240	226-261	35	15	9	60
21	PpSSR 27	Tri	151	147-161	14	5	5	100
22	PpSSR 28	Di	299	290-310	20	9	7	78
23	PpSSR 29	Di	378	360-400	40	19	10	53
Total						**270**	**168**	**62.2**

Table 5. Size of the private alleles (base pair) at each locus for *P. pinnata* genotypes

Sl. No.	locus	Private alleles in genotypes
1	SSR02	K_{18}(129), K_{15}(136), K09(139), K_{06}(143), K_{12}(151), K_{06}(155), K_{26}(158), K_{23}(160), K_{26}(162), K_{22}(163), K_{22}(164)
2	SSR03	K_{06}(296), K_{03}(298), K_{11}(304), K_{02}(308), K_{17}(325), K_{18}(326), $_{K15(}$331), K_{18}(333), K_{18}(337)
3	SSR04	K_{19}(293), K_{24}(298), K_{27}(312), K_{14}(319), K_{04}(320), K_{13}(323), K_{12}(329), K_{12}(331)
4	SSR05	K_{20}(150), K_{07}(157), K_{14}(169), K_{16}(173), K_{23}(174), K_{18}(176), K_{18}(177), K_{19}(180), K_{17}(194), K_{23}(198), K_{16}(202), K_{12}(203),K_{06}(206), K_{13}(207),
5	SSR06	K_{23}(297), K_{09}(301), K_{06}(303), K_{06}(307), K_{07}(315), K_{03}(327), K_{18}(328), K_{13}(330), K_{11}(333), K_{16}(339), K_{23}(341), K_{12}(345),K_{15}(350), K_{10}(353),
6	SSR07	K_{26}(211), K_{22}(212), K_{22}(213), K_{25}(231), K_{15}(238), K_{24}(240), K_{11}(241), K_{11}(242), K_{10}(244), K_{18}(245), K_{15}(251), K_{11}(257)
7	SSR08	K_{14}(297), K_{03}(305), K_{22}(308), K_{20}(313), K_{27}(314), K_{02}(316), K_{20}(319), K_{19}(321), K_{11}(326), K_{17}(334), K_{10}(339), K_{11}(347)
8	SSR09	K_{03}(386), K_{10}(387), K_{12}(390), K_{09}(392), K_{19}(412)
9	SSR10	K_{02}(300), K_{21}(301), K_{07}(306), K_{04}(308), K_{24}(317), K_{18}(321), K_{22}(322), K_{11}(324), K_{11}(328)
10	SSR11	K_{02}(190), K_{20}(195), K_{25}(199), K_{17}(201), K_{23}(204), K_{17}(206), K_{11}(208), K_{02}(209), K_{07}(210), K_{25}(213), K_{12}(221), K_{23}(223), K_{02}(224), K_{03}(225), K_{09}(228), K_{20}(232), K_{25}(233), K_{18}(250), K_{17}(252), K_{17}(256), K_{14}(259), K_{13}(261), K_{27}(262)
11	SSR12	K_{02}(86), K_{04}(90), K_{25}(92), K_{13}(105), K_{11}(107), K_{26}(108), K_{02}(109), K_{09}(111), K_{24}(114), K_{10}(116), K_{19}(130), K_{03}(131),K_{24}(132), K_{14}(137),
12	SSR13	K_{10}(241), K_{23}(242), K_{16}(256), K_{15}(270), K_{21}(272), K_{13}(276), K_{09}(281), K_{19}(284), K_{16}(287), K_{25}(289), K_{23}(294), K_{14}(306)
13	SSR14	K_{24}(307), K_{23}(310), K_{26}(311), K_{19}(312), K_{26}(313), K_{19}(317), K_{22}(318), K_{20}(320), K_{16}(328), K_{12}(337), K_{11}(339), K_{04}(342), K_{04}(345), K_{04}(351), K_{12}(357), K_{03}(359)
14	SSR15	NO PRIVATE ALLELE
15	SSR16	K_{07}(128), K_{02}(134), K_{06}(135), K_{18}(136), K_{17}(137), K_{25}(141), K_{26}(142), K_{11}(155), K_{07}(158), K_{26}(159), K_{19}(161), K_{07}(162), K_{04}(163), K_{04}(165), K_{19}(181), K_{12}(182), K_{17}(184), K_{25}(185), K_{17}(186), K_{12}(189), K_{15}(191), K_{26}(193), K_{06}(195)
16	SSR17	K_{07}(142), K_{16}(155), K_{13}(167), K_{16}(171)
17	SSR18	K_{24}(239), K_{23}(241), K_{07}(256), K_{09}(282), K_{10}(288)
18	SSR19	K_{12}(166), K_{21}(167), K_{25}(168), K_{15}(171), K_{26}(172), K_{23}(174)
19	SSR21	K_{20}(296), K_{04}(308), K_{15}(311), K_{11}(313), K_{19}(321), K_{14}(322), K_{14}(323), K_{04}(325), K_{14}(326), K_{12}(330)
20	SSR22	K_{20}(222), K_{12}(226), K_{21}(230), K_{09}(232), K_{25}(234), K_{22}(243), K_{23}(270), K_{23}(272)
21	SSR27	K_{20}(140), K_{26}(146), K_{13}(170), K_{13}(173)
22	SSR28	K_{04}(284), K_{12}(285), K_{12}(286), K_{04}(288), K_{11}(290), K_{07}(301), K_{09}(313), K_{17}(317)
23	SSR 29	K_{12}(360), K_{26}(361), K_{25}(366), K_{06}(385), K_{14}(387), K_{11}(390), K_{04}(391), K_{11}(392), K_{20}(394), K_{13}(395), K_{12}(396), K_{03}(399),K_{18}(400), K_{04}(404), K_{12}(405), K_{03}(406), K_{03}(407), K_{02}(409), K_{04}(411), K_{25}(412), K_{02}(413), K_{11}(414), K_{25}(416), K_{11}(424)

In addition, there were a number of loci PpSSR08, PpSSR09, PpSSR12, PpSSR13, PpSSR14 and PpSSR16 which generated private allele in K_{19} genotype (Table 5). These private allele generating loci could be further multiplexed to detect the presence of the mentioned genotype in a single reaction.

The analysis of repeat units under each motif class revealed a varying range of repeat units in each of the classes of repeat motifs. It was observed that, in tri-nucleotide motif, repeat units ranged from 2-24; in dinucleotide motif, from 13-22 and in mononucleotide units ranged from 29-30. Further analysis of the number of repeat units in every class of the SSRs, especially mono, di and tri nucleotides, showed that the number of the microsatellites decreased with increasing repeat unit. Similar result was observed by Varshney et al. [31]. These results clearly indicate the effect of increased stringency of parameters which were maintained during this study to retain the polymorphism level and utility of the SSRs as markers because the probability of polymorphism increases with increasing length of SSRs and, a higher number of repeat followed by shorter stretches would be beneficial for marker development.

Null alleles are those that fail to amplify in a PCR, either because the PCR conditions are not ideal or the primer-binding region contains mutations that inhibit binding. In this study, primers PpSSR19 could not amplify reported products in all genotypes. In the present study, detected null alleles (non-amplifying alleles) that may have been due to mutation at the priming sequence. Similar result was also observed by Callen et al. [32] in the parents of the 40 CEPH (Centre d'Etude du Polymorphisme Humain) families. In contrast, Moriguchi et al. [33] detected null alleles at 12 out of 34 loci developed (35.3%) in a segregation analysis, and deduced that the high rate of null allele detection was caused by a high mutation rate at the priming sequences in *C. japonica*. Low rate of null allele detection in the present study suggests that the high mutation rate at priming sequences is not pandemic in this species. However, requires further intensive investigation.

The present study showed newly developed microsatellite markers revealed a lower rate of null allele detection than the microsatellite markers developed by Moriguchi et al. [32]. Therefore, these markers are likely to be valuable tools, not only for genetic mapping, but also for analyses of population genetics and reproduction ecology in natural populations [34,35].

3.4 Analysis of Genetic Diversity

Genetic diversity indicated by Shannon's index varies- I (mean value in each population 0.00 - 0.3781) and Nei's mean estimates of diversity- H (from 0.00 to 0.4163) in the present study can be considered high since the values were equal to or higher than 0.40. The values found are similar to those obtained for other tree species *Populus tremuloides* (0.58 to 0.69) [36], *Fitzroya cupressoides* (0.42 to 0.56) [37] and *Swietenia macrophylla* (0.41 to 0.27) [38]. The average genetic diversity (H) obtained is somewhat higher than those found for other species e.g. *Trichilia pallida* Swartz, ranged from 0.27 to 0.33 [39] and *Aspidosperma polyneuron*, diversity averaged 0.28 [40].

The estimates of diversity in *Pongamia pinnata* through SSR studies was found to be akin/similar to other important oil yielding species Sesame. In the case of SSR data on Sesame plant, the expected heterozygosity (*He*) and observed heterozygosity (*Ho*) ranged from 0.12 to 0.48 (mean, 0.29) and 0.00 to 0.30 (mean, 0.15), respectively [41].

The UPGMA analysis revealed some important points in affinity and relationship of the 24 identified superior genotypes from eastern India. According to the UPGMA, the genotypes were divided into two subgroups: - one sub group comprised of ten genotypes i.e. K_3, K_{20}, K_{22}, K_{23}, K_{27}, K_{12}, K_{21}, K_{19}, K_{25}, K_{26} and the other sub group contained rest of the genotypes (Fig. 5). The highest and lowest oil yielding genotypes separated from each other but within the same sub group. The constructed dendrogram demonstrate that there is a considerable differentiation among the genotypes. Schaal et al. [42] suggested that historical relationships have been found to contribute populations to have similar genetic structure like that of sharing common ancestry. Therefore, there may be similar gene pools initially established in the demes during their establishment and then similar pattern of selection pressure operates on them.

Clustering of individuals from same agroclimatic in different clusters indicate high genetic variation within population which may be attributed to the use of different seed sources, mutation and/or

breeding system. This is expected for out-crossing species and it also agrees with other similar studies which had similar observations [43]. Since *P. pinnata* is a predominantly out-crossed species, it was expected to maintain most variations within populations than between populations. Several studies have reported similar observations in other plant species [44,45] although some other studies were able to find separation of genotypes based on geographic origin [46-48]. Individuals from different geographical locations are expected to be genetically different due to varying selection pressure that allow fixation of a particular character. The total genetic variation of the species is likely to be distributed among populations as the impact and direction of natural selection varies from one population to another due to environmental variation and genetic drift [49].

Based on the results it can therefore be concluded that, selection of materials for improvement of individuals and conservation of *P. pinnata* genetic resource can be achieved by considering individuals from wide geographical area in eastern India which is the native range of the species.

From the Nei's analysis it was found that maximum diversity exists between the full sib genotypes K_{10} and K_2 (Table 6). K_{16} and K_{14} are the two genotypes which are genetically identical. In plant breeding programmes, the most diverse genotypes are used in the hybridization programme. Thus, the genotypes (K_2 and K_{10}) which are more diverse could be used further in hybridization programme.

Analysis of molecular variance (AMOVA) was performed to assess the variation among and within the genotypes, using the genetic distance matrix generated by SSR markers. The partitioning of the genetic variability among the twenty four genotypes is shown in Table 7. In full sib genotypes, the analysis of molecular variance showed a significant amount of differentiation (77.92%) among the population. But with respect to overall population showed 44.16% of total variability.

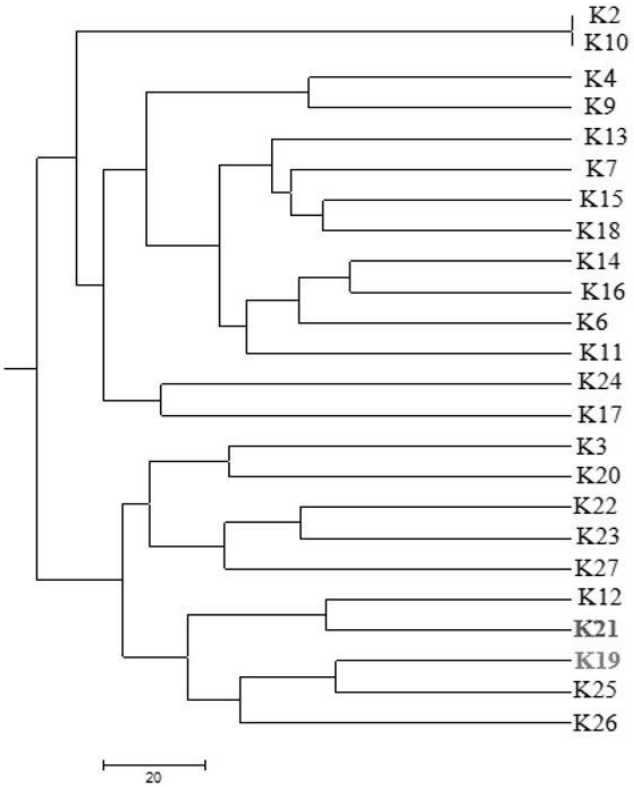

Fig. 5. Dendrogram based on Nei's analysis of genetic distance and genetic similarity in full sib genotypes

Table 6. Nei's analysis of genetic distance and genetic similarity in full sib genotypes. Nei's genetic identity (above diagonal) and genetic distance (below diagonals)

POP	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	****	0.1662	0.1720	0.1403	0.2201	0.1635	0.2871	0.0297	0.0755	0.1684	0.0768	0.1756	0.2871	0.1189	0.0615	0.1360	0.2195	0.2379	0.3568	0.2634	0.1879	0.0000	0.0743	0.0994
2	1.7945	****	0.1876	0.1263	0.1027	0.1338	0.1511	0.0892	0.2569	0.2245	0.2151	0.1610	0.2418	0.1487	0.1537	0.1209	0.0293	0.0595	0.1041	0.0585	0.2457	0.0577	0.0446	0.1562
3	1.7604	1.6734	****	0.1597	0.2581	0.3077	0.2970	0.2615	0.2189	0.2614	0.2067	0.1211	0.1094	0.0769	0.0636	0.1563	0.1060	0.3230	0.1538	0.2271	0.2542	0.0447	0.1538	0.0735
4	1.9637	2.0691	1.8343	****	0.3271	0.2209	0.1824	0.1657	0.1965	0.3780	0.3139	0.1359	0.2245	0.1519	0.2283	0.1123	0.2175	0.0690	0.1933	0.2039	0.1342	0.2142	0.0967	0.1319
5	1.5137	2.2758	1.3544	1.1176	****	0.3465	0.2788	0.0722	0.1321	0.1499	0.1343	0.1705	0.0587	0.0433	0.2238	0.1907	0.3127	0.1877	0.2887	0.3127	0.1263	0.1960	0.1588	0.1241
6	1.8107	2.0114	1.1788	1.5100	1.0599	****	0.4163	0.1755	0.2527	0.1657	0.2570	0.3024	0.1635	0.1902	0.1361	0.2825	0.2160	0.1609	0.2340	0.2304	0.2559	0.0851	0.2487	0.1677
7	1.2480	1.8898	1.2139	1.7014	1.2773	0.8764	****	0.1635	0.0453	0.0982	0.1844	0.2342	0.1511	0.1635	0.1537	0.4080	0.2927	0.2825	0.4014	0.2342	0.3179	0.1153	0.2527	0.1278
8	3.5155	2.4169	1.3413	1.7976	2.6285	1.7399	1.8107	****	0.2379	0.2347	0.3931	0.1872	0.3122	0.1316	0.1814	0.1635	0.1152	0.1316	0.0439	0.2016	0.1848	0.1418	0.1316	0.1397
9	2.5830	1.3592	1.5192	1.6273	2.0245	1.3754	3.0938	1.4360	****	0.2666	0.2151	0.1903	0.1662	0.1933	0.1997	0.0151	0.1610	0.0446	0.1635	0.2488	0.1301	0.1153	0.1635	0.2414
10	1.7814	1.4937	1.3419	0.9730	1.8978	1.7976	2.3204	1.4493	1.3219	****	0.3425	0.1903	0.2245	0.1243	0.1570	0.0281	0.0272	0.0690	0.1243	0.1495	0.0805	0.1071	0.0690	0.0923
11	2.5662	1.5366	1.5766	1.1585	2.0078	1.3586	1.6907	0.9338	1.5366	1.0715	****	0.3869	0.2766	0.1814	0.1094	0.0615	0.0744	0.0605	0.0756	0.1339	0.1176	0.0879	0.1361	0.1878
12	1.7394	1.8264	2.1108	1.9957	1.7688	1.1960	1.4517	1.6756	1.6594	1.6592	0.9495	****	0.1317	0.1296	0.1488	0.1610	0.1418	0.0576	0.1440	0.1701	0.2099	0.0558	0.1440	0.1100
13	1.2480	1.4198	2.2124	1.4937	2.8355	1.8107	1.8898	1.1641	1.7945	1.4937	1.2853	2.0271	****	0.3419	0.1229	0.1058	0.1024	0.0743	0.2230	0.2488	0.1734	0.1153	0.1041	0.2414
14	2.1292	1.9060	2.5651	1.8847	3.1394	1.6599	1.8107	2.0276	1.6437	2.0853	1.7070	2.0433	1.0731	****	0.1058	0.0297	0.1440	0.1170	0.2194	0.1296	0.1848	0.1135	0.0585	0.2655
15	2.7894	1.8731	2.7552	1.4770	1.4969	1.9946	1.8731	1.7070	1.6107	1.8517	2.2130	1.9050	2.0962	2.2460	****	0.2919	0.2232	0.1209	0.1663	0.2232	0.0882	0.3225	0.1965	0.2022
16	1.9952	2.1130	1.8557	2.1869	1.6568	1.2642	0.8966	1.8107	4.1924	3.5732	2.7894	1.8264	2.2465	3.5155	1.2312	****	0.2634	0.2527	0.2527	0.3073	0.2601	0.1009	0.1784	0.1704
17	1.5163	3.5312	2.2443	1.5257	1.1626	1.5325	1.2286	2.1611	1.8264	3.6051	2.5981	1.9537	2.2784	1.9380	1.4995	1.3340	****	0.1872	0.3168	0.3402	0.1959	0.1256	0.1440	0.0688
18	1.4360	2.8223	1.1300	2.631	1.6730	1.8269	1.2642	2.0276	3.1100	2.6731	2.8056	2.8543	2.5992	2.1454	2.1124	1.3754	1.6756	****	0.1609	0.0720	0.0995	0.0284	0.1024	0.0699
19	1.0306	2.2627	1.8719	1.6435	1.2422	1.4523	0.9128	3.1262	1.8107	2.0853	2.5824	1.9380	1.5006	1.5168	1.7940	1.3754	1.1495	1.8269	****	0.3744	0.2133	0.1418	0.1902	0.1677
20	1.3340	2.8380	1.4822	1.5902	1.1626	1.4680	1.4517	1.6015	1.3911	1.9004	2.0103	1.7714	1.3911	2.0433	1.4995	1.1798	1.0782	2.6311	0.9825	****	0.3079	0.1536	0.1008	0.1926
21	1.6721	1.4038	1.3697	2.0084	2.0692	1.3629	1.1460	1.6883	2.0398	2.5192	2.1409	1.5609	1.7521	1.6883	2.4285	1.3467	1.6299	2.3074	1.5452	1.1779	****	0.0689	0.1280	0.1494
22	0.0000	2.8533	3.1068	1.5409	1.6298	2.4640	2.1601	1.9532	2.1601	2.2340	2.4310	2.8852	2.1601	2.1763	1.1318	2.2937	2.0743	3.5626	1.9532	1.8736	2.6748	****	0.2269	0.0677
23	2.5992	3.1100	1.8719	2.3366	1.8401	1.3916	1.3754	2.0276	1.8107	2.6731	1.9946	1.9380	2.2627	2.8385	1.6269	1.7237	1.9380	2.2789	1.6599	2.2946	2.0560	1.4832	****	0.1677
24	2.3084	1.8564	2.6108	2.0257	2.0865	1.7857	2.0571	1.9680	1.4211	2.3824	1.6726	2.2068	1.4211	1.3261	1.5985	1.7694	2.6768	2.6611	1.7857	1.6472	1.9011	2.6921	1.7857	****

Table 7. Partitioning of genetic variability among the different source of variation (AMOVA design) in *P. pinnata*

Source of variation	d.f.*	Sum of squares	Variance components	Percentage variation
Among populations	23	1105.938	8.01404 Va	77.92
Among individuals within populations	48	0.000	-2.27083 Vb	-22.08
Within individuals	72	327.000	4.54167 Vc	44.16
Total	143	1432.938	10.28487	

d.f. = Degree of freedom*
Significance test (1023 permutations) at Significance Level = 0.05
P-value = 0.00000 for all variance component.

Table 8. Comparison of allelic differences in between the highest oil yielding and lowest oil yielding genotypes

Locus	Type of repeat (nucleotides)	Genotypes K_{19} (allele size) and types of alleles	Genotypes K_{21} (allele size) and types of alleles
SSR02	Di	145/ 145(Homozygous)	145/ 145(Homozygous)
SSR03	Di	302/ 302(Homozygous)	300/ 300(Homozygous)
SSR04	Mono	293/ 293(Homozygous)	309/ 309(Homozygous)
SSR05	Tri	151/ 180(Heterozygous)	155/ 186(Heterozygous)
SSR06	Tri	300/ 322(Heterozygous)	300/ 325(Heterozygous)
SSR07	Di	223/ 223(Homozygous)	217/ 217(Homozygous)
SSR08	Di	317/ 317(Homozygous)	300/ 300(Homozygous)
SSR09	Mono	395/ 395(Homozygous)	407/ 407(Homozygous)
SSR10	Di	310/ 310(Homozygous)	301/ 301(Homozygous)
SSR11	Tri	197/ 246(Heterozygous)	217/ 217(Homozygous)
SSR12	Di	97/ 124(Heterozygous)	101/ 101(Homozygous)
SSR13	Tri	251/ 298(Heterozygous)	247/ 300(Heterozygous)
SSR14	Di	321/ 321(Homozygous)	329/ 329(Homozygous)
SSR15	Di	195/ 195(Homozygous)	195/ 195(Homozygous)
SSR16	Di	146/ 181(Heterozygous)	146/ 167(Homozygous)
SSR17	Di	149/ 169(Heterozygous)	149/ 149(Homozygous)
SSR18	Tri	249/ 275(Heterozygous)	247/ 272(Heterozygous)
SSR19	Di	?/ ?	167/ 167(Homozygous)
SSR21	Di	300/ 300(Homozygous)	294/ 294(Homozygous)
SSR22	Di	237/ 254(Heterozygous)	230/ 240(Heterozygous)
SSR27	Tri	149/ 149(Homozygous)	148/ 148(Homozygous)
SSR28	Di	291/ 291(Homozygous)	295/ 295(Homozygous)
SSR29	Di	369/ 369(Homozygous)	362/ 373(Heterozygous)

Private alleles were also found in these *P. pinnata* genotypes. Private alleles (which are only present in one genotype and absent from all others tested) were suspected to have accelerated the genetic differentiation in these populations. K_{11} had relatively more private alleles with high frequency. At locus PpSSR20, twenty four private alleles were founded. The locus PpSSR15 did not show any private alleles. In the genotype K19, number of locus showed private alleles. These private alleles were further used for this high oil yielded genotype characterization and unique marker development (Table 5). On the basis of allele size, the high oil yielding and low oil yielding genotypes show significant variation (Table 8).

4. CONCLUSION

In conclusion, we isolated the first set of twenty three polymorphic microsatellite loci and characterized across twenty four *P. pinnata* accessions. Despite the modest number of genotypes analyzed, the results indicate that the

new microsatellite loci developed in this study will serve as a very useful tool for genetic diversity analysis, clonal identification and conservation in *P. pinnata* germplasm. High level of genetic variation was found among different genotypes of *P. pinnata*. Both molecular and oil content (biochemical) markers appeared useful in analyzing the extent of genetic diversity in *Pongamia* and the result of these analyses will help to better understand the genetic diversity and relationship among populations.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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