

Genetic Diversity Analysis of Selected Sengon(*Paraseranthes Falcataria*) Clones Using SSR Markers Dirived from Acacia Species

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Abstract

Understanding of genetic diversity among sengon(*paraseranthes falcataria*) is very important for selection and improvement of its breeding. This study determined the genetic diversity among the previous preferred 36 clones which selected from 6 districts. Of 8 *Paraserianthes falcataria* SSR markers and 76 SSR markers selected from *Acacia* species, only 13 markers showed polymorphic pattern and amplified a total of 61 alleles. The polymorphism information content (PIC) value at a locus ranged from 0.099 to 0.799. The average number of alleles per locus ranged from 2 to 9 with an average of 4.92. Six SSR markers were highly informative and had PIC values above 0.60. The results demonstrated that the SSR DNA-markers developed from *Acacia* species could be successfully used to differentiate genotypes of *P. falcataria*. Cluster analysis of SSR data using UPGMA separated 36 clones into 2 divergent clusters at similarity coefficient of 0.56. The diverse clones identified in the study would be useful for developing intraspecific hybrids to exploit hybrid vigour and for commercial cultivation to broaden genetic base. Meanwhile, the DNA fingerprints obtained for each clone can be used for identification of breeding population and accelerate research of *P. falcataria* breeding.

Keywords: *Paraseranthes Falcataria*; SSR; Genetic Diversity; Polymorphism Information Content

1 INTRODUCTION

Paraseranthes falcataria(L.) Nielsen, also known as sengon, is native to Indonesia, Papua New Guinea, Solomon Islands and Australia. Now it is widely planted throughout the tropica especial in south-east Asia including Philippines, Vietnam, Thailand and so on owing to its very fast growth, its ability to grow on a variety of soils and its acceptable quality of wood for the panel and plywood industries(Krisnawati et al., 2011). Meanwhile, it is possible to select a plus tree with high-density wood at a relatively early stage(Ishiguri et al., 2007; Osawa and Kojima, 2006). Besides, as a nitrogen-fixing species, *P. falcataria* is also commonly planted for reforestation to improve soil fertility and mixed plantations and is a good tropical trees for research aluminum resistance mechanism(Osawa and Kojima, 2006; Siregar et al., 2007).

P. falcataria were introduction in china by sun yat-sen university in early 1940s, and developed systemic seedlots/families experiments in 1998(Caixi, 1965; Zheng et al., 2003). Meanwhile, they developed a series of experiments including seedlots/families, cultivation techniques test(Xiaozhen, 2003; YAN et al., 2012). Regretfully, most of planting tree were secondary provenance and lacked proper provenance records, which prevented the further selection and breeding. And some farmers have long been actively planting *P. falcataria* trees on private or community land in Huizhou and Guangzhou, Guangdong province. However, it was possible to select the superior clones by using more than 50 ha *P. falcataria* forest. Based on these, we selected 36 superior clones with branch-free stem and good growth rates. Unfortunately, these superior clones origin were unknown totally. Hence, it was necessary to develop molecular markers in *P. falcataria* to analyze the genetic variation and genetic diversity for

further breedings. However, Molecular markers for *P. falcataria* had seldom been reported and only 8 microsatellite markers were isolated (Y Saito, 2014; Yuskianti and Shiraishi, 2010).

SSR marker which was known as its more polymorphic and more consistent results had been proved as the most informative marker in diversity analysis. Meanwhile, SSR markers can sometimes be transferable among related species, providing an opportunity to study one related species diversity analysis under low cost (Zhang et al., 2005). Some success had been observed in crops and cereals (Butcher et al., 2000; Erayman et al., 2014; Fan et al., 2013; Gao et al., 2012; Reddy et al., 2010; Wang et al., 2015; Zhang et al., 2005). Additionally, inter-generic transferability have been verified such as transferring rice microsatellites to bamboo species (Chen et al., 2010). Therefore, the main goals of this study were to investigate a large number of SSR primers from *Acacia* species to find the most useful loci for genetic studies in *P. falcataria*. Transferability and polymorphism information for each primer were analyzed and selected microsatellite markers will be explored the genetic diversity and structure analyses of 36 *P. falcataria* clones. Meanwhile, These results will open opportunities to further improve the efficiency of breeding programs and broadened the genetic base of breeding populations of *P. Falcataria*.

2 MATERIALS AND METHODS

2.1 Sample Collection

Leaves from 36 clones of *P. falcataria* which obtained from previous selected excellent clones in Guangdong provinces of China were used in this experiment. The details listed in table 1.

2.2 DNA Extraction, SSR Primer and PCR Amplification

Genomic DNA was extracted from *P. falcataria* leaves following the instruction of plant DNA extraction Kit (Aidlab, Beijing, China). Purified DNA was quantified by electrophoresis in 1.0% agarose gel, which were absence of RNA band and no smear in the gel. Meanwhile, the purity and concentration of DNA samples were evaluated using a NanoDrop 2000 spectrophotometer (NanoDrop, Thermo Scientific). Samples with concentrations greater than 100 ng.µL⁻¹ and A260/A280 between 1.6 and 1.8 were used for diluted 50 ng.µL⁻¹ with sterile ddH₂O for further DNA amplification.

Then, we tested the 15 SSR markers used in *Acacia* hybrid (*A. mangium* × *A. auriculiformis*) (Ng et al., 2005), as well as 33 additional SSR markers used in *A. mangium* (Butcher et al., 2000), and 20 SSR markers used in *A. auriculiformis* × *A. mangium* (Le et al., 2016), and 8 SSR markers developed in *P. falcataria* itself (Saito et al., 2014). To carry out the analyses in all the genetic materials, primers showing an clear amplification product length ranging between 80 bp and 400 bp under an optimal annealing temperature were selected for further analysis. Then SSR primers available in the literature were used in the 36 *P. falcataria* clones. For the latter system, each forward primer was 5' -tailed with the M13 forward consensus sequence (5'-CGTTGTAAAACGACGGCCAGT-3'). The M13-tailed forward primers were then used in combination with a standard M13 primer 6-FAM-labeled at its 5' -end (Nicot et al., 2004). PCR reactions for the SSR markers were made in 20 µL containing 1×PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.04 units Taq DNA polymerase (Takara), 0.2 µL forward primer, 0.8 µL reverse primer, 0.8 µL M13-labelled primer (of 100 nM of each of the primer pair) and 1 µL of template DNA were added and the remaining volume was completed with sterile ddH₂O. Amplification was performed using 96 Veriti Applied Biosystems thermocycler programmed for initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, 56 °C for 45 s, and extension at 72 °C for 45 s, followed by 8 cycles of 94 °C for 30 s, 53 °C for 45 s and 72 °C for 45 s, then a final extension at 72 °C for 7 min and final hold at 4 °C for temporal storage. All primer sets used in this study were amplified using the same cycling conditions. The program consisted of PCR products were each added to HIDi formamide with A LIZ-500 size standard and subjected to capillary electrophoresis with ABI3730 DNA genetic analyser for fragment analysis. Allele calls were made using GENEMAPPER software v.3.7 (Applied Biosystems).

2.3 Polymorphic Analysis

Data generated by GENEMAPPER software v.3.7 (Applied Biosystems) was transferred to excel and analyzed for

various genetic parameters which included number of alleles per locus, heterozygosity(He), polymorphic information content(PIC) using GenALEx(Peakall and Smouse, 2006). Cluster analysis was used to group the clones and to construct a dendrogram using NTSYSpc version 2.10e software(Rohlf, 2005). The similarity matrix representing the Co-efficient was used to cluster the clones using molecular data based on UPGMA(Unweighted Pair Group Method with Arithmetic Mean). A cluster plot of the 36 clones was constructed using SAHN(Sequential Agglomerative Hierarchic Nonoverlapping clustering) and Tree Plot procedures of the NTSYSpc version 2.10e software.

3 RESULT AND DISCUSSION

3.1 Characteristics of SSR Markers

Seven samples from 7 district screening SSR loci from a total of 76 published primers including 68 Acacia primers and 8 *P. falcata* primers. 20 loci showed clear band amplification(Figure 1), in which 13 loci including 12 SSR loci originated from Acacia(17.7%) chosen on the reliable and polymorphic production of interpretable microsatellite DNA profiles in a test panel. The 13 SSR primers identified 61 alleles among the 36 *P. falcata* clones. DNA amplification profile of these clones with representative marker P28 is presented in Figure 2. These results verified that the transferability of SSR markers among *A. mangium* and *A. mangium* × *A. auriculiformis*. Meanwhile, transferability of *A. mangium* and *A. mangium* × *A. auriculiformis* SSR markers to *P. falcata* was 22.9% and 12.1%, respectively. The number of alleles scored across SSR loci ranged from 2 to 9, with an average of 4.92 alleles. The PIC estimated for all loci ranged from 0.799 to 0.099 with an average of 0.507. Seven SSR loci had PIC values less than 0.60. However, the remaining six SSR makers had potential to detect differences among the 36 clones. Of 6 markers, Primer 10 amplified 9 alleles and had the highest PIC value of 0.799 and hence had high power of discrimination (Table 2). Expected heterozygosity (He) values, as a measure of allelic diversity at a locus, varied from 0.700 to 0.182 with an average of 0.431.

TABLE 1 LIST OF 36 *P. FALCATA* CLONES USED IN THE STUDY

Clone	Source
Da1, Da2	Datian, Boluo
A1, A2, A3, A4, A6, A7, A8, A9	Chiling, Zengcheng
S1, S4, S5, S6, S7	Shajing, Longmen
B2, B4, B6, B7, B8	Baima, Huidong
D1, D11, D12, D18, D19, D21, D22	Baihua, Huidong
11, 12	Huizhou
TL01, TL11, TL12, TL13, TL14, TL16, TL17, TL18	Tianliaobei, Boluo

3.2 Genetic Relationships

12 SSR markers derived from Acacia species were successfully transferred to *P. falcata* to detect genetic diversity. The transferred markers and 1 SSR markers stem from *P. falcata* were used to construct dendrograms to identify how each marker system formed the genetic similarity among the 36 clones. The results showed that the 13 microsatellite markers distinguished all 36 clones. The Jaccard genetic similarity coefficients values were between 0.46 and 0.98. The dendrogram constructed using the UPGMA clustering algorithm based on SSR data matrices grouped these clones into two major clusters at similarity coefficient of 0.56 (Fig. 1). The selected SSR markers did not cluster 36 clones into different source groups. It speculated that all clones which selected from farmer's planting actually stem from different provenances. The result implied it was necessary to identify these clones genetic diversity to further breeding. However, a few clones could be clustered one groups such as S1, S4, S5, S6 and S7 selected from Shajing belong to the first subgroup of cluster I. Meanwhile, highest genetic similarity coefficient in this subgroup was 0.98 between S1 and S7, while these two clones had similarity coefficient of 0.95 with S4. Besides, TL13 and TL17 showed high level of similarity with coefficient value 0.92 were also found.

Of two clusters formed, cluster I which contained 31 clones was more than cluster II. These clones were selected from different sources were clustered into a group. It was further subgrouped into four groups at similarity coefficient of 0.68. In the first subgroup of cluster I, there were 25 clones which mainly included five clones

including A1, A2, A7, A8 and A9 from Chiling, Zengcheng, four clones (TL11, TL13, TL17, TL18) from Tianliaobei, Boluo, five clones (S1, S4, S5, S6, S7) from Shajing, Longmen, four clones (D11, D12, D18, D22) from Baihua, Huidong, three clones (B6, B7, B8) from Baima, Huidong, two clones (Da1, Da2) from Datian, Boluo, two clones (H1, H2) from Huizhou. The second subgroup comprised of three clones only A6, B4, TL14. Besides, the third subgroup of cluster I consisted of A3 and TL12 with similarity coefficient of 0.71, while the fourth subgroup consisted of TL01 alone. Cluster II comprised of D21, A4, D19, B2, TL16 and D1 clones. The markers developed in this study may also be used to determine where these clones originated. Meanwhile, these markers may also be useful for distinguishing *P. falcata* from other *Paraseranthes*. Hence, the SSR markers generated useful information that will assist in identifying clones to include in the breeding program. The microsatellite markers developed in this study will be useful in *P. falcata* breeding and improvement programs in the future.

TABLE 2 PRIMER SEQUENCES, ANNEALING TEMPERATURES (T_m) AND THE SOURCE OF PRIMERS USED FOR *P. FALCATA*

Primer	Sequence(5'-3')	T _m (°C)	Original code	Source species	Reference
P4	TAGGCTAATGGTCATATTCCTAG AGAGATAGGGGTACACACTAAAAAAC	47	Am041	<i>A. mangium</i>	(Butcher et al., 2000)
P8	CCTCATGTCCTTGAATGTCAC GACTAACCCACAAGGAAGAGTTAC	47	Am352	<i>A. mangium</i>	(Butcher et al., 2000)
P10	AGACTTCATAAATAAGATGGAAGAGG ATGCCAAATTTCTTATTGGAG	49	Am384	<i>A. mangium</i>	(Butcher et al., 2000)
P12	AATCCTTCCGAAAGTTATACATGG GCACTTGTAAGTCGGAAGTGC	45	Am389	<i>A. mangium</i>	(Butcher et al., 2000)
P14	CGGATGAGAAGTGGCATTAGG GCCATCCAGAGTTGAAATTAGC	49	Am396	<i>A. mangium</i>	(Butcher et al., 2000)
P15	AAGCACTTCTAGATTGGCAGC CAATAATCTAGAACTACGGGACTTG	45	Am400	<i>A. mangium</i>	(Butcher et al., 2000)
P21	TGGGTATCACTTCCACCATT AGGCTGCTTCTTTGTGCAGG	47	Am465	<i>A. mangium</i>	(Butcher et al., 2000)
P25	ATGAGTTTGACCCGACTTGA TGCGTAACACGATTATCCCT	49	Am522	<i>A. mangium</i>	(Butcher et al., 2000)
P27	ACAGTGGTATGATGGGAGTG CACTAACGTCACAATGATCG	50	AH2-6	<i>A. mangium</i> × <i>A. auriculiformis</i>	(Ng et al., 2005)
P28	CGTCTCATCGATCTTCTTTC GGAGGCATAACATCAAACAT	50	AH2-9	<i>A. mangium</i> × <i>A. auriculiformis</i>	(Ng et al., 2005)
P30	AGGGATATCGGATGCTTACT AAAGATGCAGCAGACCTATC	55	AH3-10	<i>A. mangium</i> × <i>A. auriculiformis</i>	(Ng et al., 2005)
P31	GGGGAGGCTCTCTCTCTCTCTCTCT GTGACCTGAGTTAGGAAGGAGC	48	AH76	<i>A. mangium</i> × <i>A. auriculiformis</i>	(Ng et al., 2005)

The 13 SSR primers were successfully developed among the 36 *P. falcata* clones, which 12 SSR loci originated from *Acacia*. The transferability was lower than that reported for the transfer of rice SSR markers to bamboo

(74.5%)(Chen et al., 2010) and for pear SSR markers cross-amplified in apple (58.2%)(Fan et al., 2013). It indicated that *Acacia* and *P. falcata* may not be that distantly related. Because transferability had been shown to be negatively correlated with evolutionary complexity and genetic distance between species(Chen et al., 2010; Heesacker et al., 2008). Besides, the lower transferability was reported for the intragenic amplification of SSR markers in the legume genera and intergeneric amplification of *acacia* species, and that in other crossgenus SSR amplification studies(Butcher et al., 2000; Erayman et al., 2014; Gao et al., 2012; Wang et al., 2015). It indicated that transferability was associated with the genera and species specific. PIC values were usually described the relative values of each marker with respect to the amount of polymorphism(Botstein et al., 1980; Grewal et al., 2014). It could be influenced by various factors including the nature of germplasm, number of SSR loci and number of clones analyzed etc(Bantte and Prasanna, 2003). In this study, seven SSR loci showed lower PIC values with less than 0.60, which may be the result of closely related genotypes and provenance distribution.

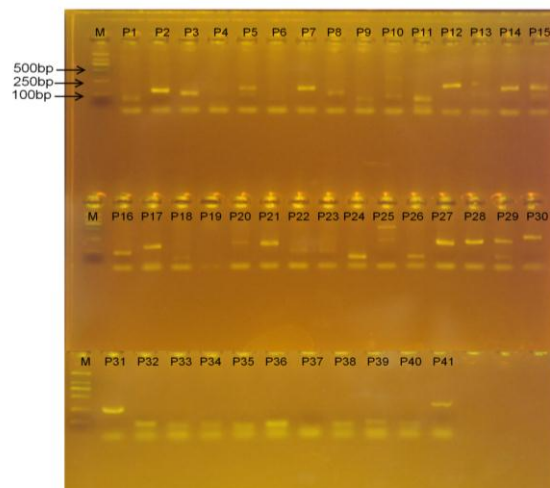


FIGURE1 AMPLIFICATION PRODUCTS SHOWED BY 2.5% AGAR OF THE SELECTED 41 PRESENTATION PRIMERS.LANE 1-41 REPRESENT DIFFERENT PRIMERS INDIVIDUALS, M IS THE DNA MARKER.

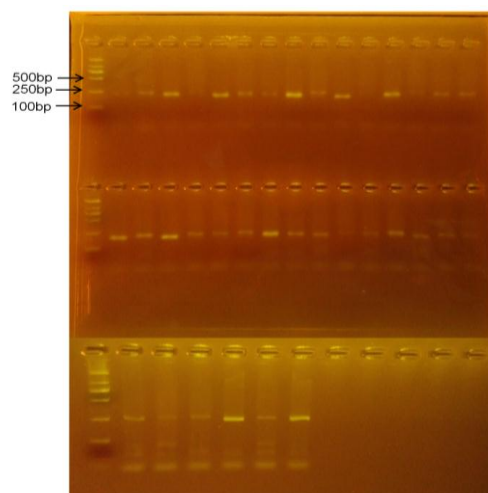


FIGURE 2 2.5% AGAR SHOWING PCR AMPLIFICATION OF 36 CLONES OF *P. FALCATARIA* WITH REPRESENTATIVE MARKER P28. LANE 1-36 REPRESENT DIFFERENT *P. FALCATARIA* CLONES INDIVIDUALS, M IS THE DNA MARKER.

13 SSR markers were used to construct dendrograms, the 36 *P. falcata* clones were grouped into two major clusters. The selected SSR markers did not cluster these clones into different source groups, which verified our previous speculation. The result implied it was necessary to identify these clones genetic diversity to further breeding. Meanwhile, high genetic similarity coefficient with over 0.95 among S1, S7 and S4 collected from the same place were happened, which suggested that they were originated from a family. These results were also happened in other plant species by SSR identified different clone(Grewal et al., 2014; Tan et al., 2015). In our previous studies, many

trees in *P. falcata* plantations die owing to disease within a few years of planting. Interestingly, TL13 and TL17 clones, A1 and A9 clones appeared to be resistant to stem canker disease by our previous experiments could be grouped into one subgroup. These results implied that these microsatellite loci linked to genes conferring resistance to disease may be used as selectable markers in future *P. falcata* improvement programs. In some plant species, microsatellite loci linked to disease resistance were identified by analyzing disease-resistant and susceptible plant samples (Palti et al., 2015; Vallejo et al., 2014; Zhang et al., 2012). However, TL12, TL16, TL18 and D1 clones showed disease susceptible plants were not clustered one group. The probability was that the SSR markers used provide insufficient resolution to discriminate between these clones. Hence, it is necessary to develop SSR primers to identify microsatellite loci linked to genes conferring resistance to disease for further. However, the microsatellite markers developed in this study will be useful in breeding and improvement programs of *P. falcata* in the future.

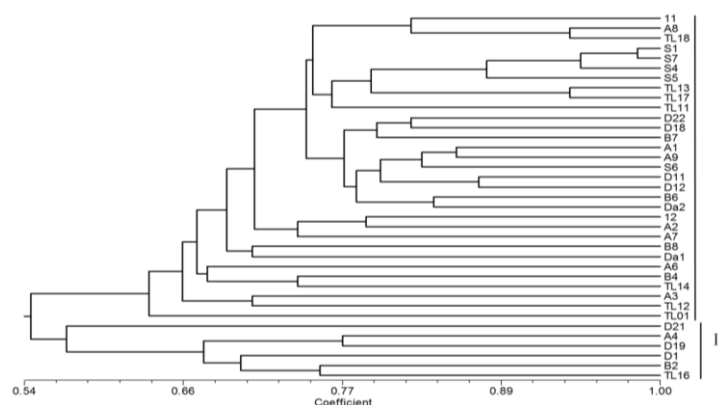


FIGURE 3 DENDROGRAM SHOWING GENETIC RELATIONSHIP AMONG 36 *P. FALCATARIA* CLONES OBTAINED USING 13 SSR MARKERS GENERATED BY UPGMA.

ACKNOWLEDGMENT

We would like to thank State Forestry Administration of the People's Republic of China for financially supporting our work through National forestry public sector research and development (R&D) project grant (201304113). Authors acknowledge technical assistance for fragment analysis by Dr. Changpin Zhou.

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