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Genetic diversity of *Neolamarckia cadamba* using dominant DNA markers based on inter-simple sequence repeats (ISSRs) in Sarawak

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ABSTRACT

Neolamarckia cadamba or commonly known as kelampayan has been selected as one of the important plantation tree species in Malaysia. Thus, the molecular characterization of this indigenous tropical tree species is needed to maintain its high quality. Inter simple sequence repeats (ISSR) markers were used in this study to determine the genetic diversity and relatedness of N. cadamba in two planted forests and six natural forests in Sarawak. Three ISSR primers had generated atotal of 239 loci, of which 32.6% - 59.4% of the loci were polymorphic among236N. cadamba treesin eight populations. The mean Shannon's diversity index (I)ranged from 0.1399 to 0.2354. The coefficient of population differentiation was low for planted forests (G_{st} = 0.0871) and natural forests (Gst = 0.2013`). Both UPGMA dendrogram and NJ-tree generated by ISSR markers had divided natural forests and planted forests into two distinct clusters. Natural forests were grouped in one cluster while planted forests were grouped in another cluster. This study shows that N. cadamba trees are closely related within its own population and its designated forest type. In future, several specific loci can be sequenced and developed into SCAR (sequence characterized amplification region) markers for tree improvement and conservation programme of N. cadamba.

Keywords: Genetic Diversity, *Neolamarckia cadamba*, Kelampayan, Inter simple sequence repeats (ISSR), Planted Forest, Tree Improvement.

INTRODUCTION

Forest plantations have become increasingly important nowadays as a result of extensive deforestation activities. Human beings have been utilizing forest trees for decades as a source of materials for providing shelter, fuel, food, fiber, medicinal plants and others. The price of industrial woodshad thus increase due to the declining availability of harvesting native forests. This phenomenon has brought to the initiation of industrial forest tree planting. In the past, reforestation involved replanting of existing wild trees regardless of the genotypic trait. To date, biotechnology approaches have been used to select tree species with targeted characteristics, such as short rotation period and higher yields at the molecular level.

DNA markers are used in molecular characterization because DNA markers can act rapidly and economically to characterize cultivars, provenances or genotypes precisely and enable the measurement of genetic relationships [1]. Some DNA marker systems that are commonly used and involved PCR amplification are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and more recently, simple sequence repeat (SSR) and inter simple sequence repeats (ISSR) [2]. They have been widely used in genetic analysis, gene mapping and genotype identification of forest tree species. ISSR-PCR has an advantage over other DNA fingerprinting methods such as it is fast, requires no radioactive handling facilities and involves minimal cost.

Polymorphism may be occurring within a species due to mutation or crossover of the chromosome [3]. Although there are no significant differences in morphology characteristics, polymorphism will still cause a species to have

different gene sequence between individuals. This may causes the cultivation of undesired trait because it has the similar morphology with the desired trait. One of the early approaches that can be done is by screening the genetic variation by using molecular DNA markers.

Hence, the main objective of this study was to determine the genetic diversity and genetic relatedness of the selected *Neolamarckiacadamba* trees from six natural forests and two planted forestsin Sarawak, Malaysia byusing ISSR-PCR markers. *N. cadamba* or locally known as Kelampayan is one of the evergreen and fast growing tropical forest tree species [4]. These characteristics have caused it to be cultivated worldwide, especially in tropical regions. In Malaysia, *N. cadamba* has been selected as one of the plantation tree species in forest rehabilitation projects due to its short rotation period which can give early commercial returns within 8-10 years.

MATERIALS AND METHODS

Plant materials: Fresh leaf samples of *N.cadamba*were collected from six natural forests (i.e., Similajau in Bintulu, Lawas, Matang, Simunjang, Mukah Hill and Niah) and two planted forests (i.e., Nanga Dap, Kanowit and Pasin, Song). Approximately 25 to 35*N. cadamba* trees were collected from each population.

Total genomic DNA isolation: DNA was extracted from the leaf samples according to the modified CTAB method [5]. The leaf was ground in the presence of liquid nitrogen and incubatedin 1 mL preheated CTAB extraction solution (2% (w/v) CTAB buffer, 100mM Tris-Cl at pH8.0, 20mM EDTA at pH8.0, 1.4M NaCl and 2% (v/v) β-mercaptoethanol) for 1 hour. Equal volume of 24:1 (v/v) chloroform/isoamyl alcohol was added and centrifuged twice before precipitated the DNA with 2/3 volume of isopropanol. The precipitate was washed with 70% ethanol, dried and resuspended in 30 µL sterile ultra-pure water. The extracted DNA sample was purified using the Wizard Genomic DNA Purification Kit (Promega, USA). The resuspended DNA was topped up with distilled ultra pure water to 600 µL and added with 3 µL of 4 mg/mL RNaseA (Invitrogen, Brazil). After incubated at 37°C for 1 hour, 200 µL of Protein Precipitate Solution was added and centrifuged, followed by incubation in 600 µL isopropanol at -20°C for 30 minutes. The precipitate was washed with 70% ethanol, dried and resuspended in sterile ultrapure water. DNA was stored at -20°C until further used.

ISSR-PCR: Three microsatellite primers namely: $(AC)_{10}$, $(AG)_{10}$ and $(GTG)_6$ were used in this project to amplify the ISSR region (Figure 1). PCR was carried out by using a Mastercycler Gradient PCR (eppendorf, Germany). DNA amplification was carried out in 25 µL reaction volume containing 1x PCR buffer (10 mM Tris-HCl at pH 8.8 and 50 mMKCl), 2.5 mM $MgCl_2$, 0.2 mM of each dNTPs (dATP, dCTP, dTTP and dGTP), 0.5 unit of *Taq* DNA polymerase (Promega, USA), 10.0 pmol/µL of primer and 2ng of genomic DNA. The thermal cycling profile was programmed at 94°C for 2 minutes as the initial denaturation step, 39 cycles of 30 seconds at 94°C, 30 seconds at 65°C for $(GTG)_6$, 57.8°C for $(AC)_{10}$ and 59.1°C for $(AG)_{10,}$ 1 minute at 72°C and final extension step at 72°C for 10 minutes.The amplification products were then subjected to 1.5% agarose gel electrophoresis at 80V for 2 hours and stained with 1X Gelstar Nucleic Acid Gel Satin (Cambrex, USA) for 30 minutes. The gel with amplification products was visualized using the UV transilluminator and documented using Geliance 200 Imaging System (PerkinElmer, USA).

Data analysis: The DNA bands produced at different loci were determined and named for each DNA sample. Banding profiles generated were converted into a binary data matrices on the basis of present (1) or absent (0) of bands. Data scoring is based on several criteria: (1) locus is assumed as independent or non-allelic, (2) there is no bias in scoring monomorphic fragments versus polymorphic fragments, (3) amplified loci are expected to be in the range of 250 bp to 1500 bp, and (4) the similarity of fragment size is assumed to be the indicator of homology. The binary matrices were used to estimate DNA polymorphism or genetic diversity and genetic relatedness of *N.cadamba*. Genetic data analysis was performed by using POPGENE version 1.32 software by assuming Hardy-Weinberg equilibrium to calculate percentage of polymorphic loci (*P*), Shannon's diversity index (*I*), and Nei's (1978) [6] coefficient of population differentiation (Gst)value. Pairwise standard genetic distance between populations based on the Nei's genetic distance [7] was calculated by using PowerMarker version 3.25 [8] to construct UPGMA dendrogram.Clustering ofall the*N. cadamba*trees in natural and planted forests was also performed based on the shared allele distance, D_{SA}[10]byusing PowerMarker version 3.25 and neighbour-joining tree was constructedby using *MEGA* version 4 [9].

RESULTS AND DISCUSSION

A total of 236 *N. cadamba* trees were analyzed in the present study. Three selected dominant markers had produced different number of ISSR bands, depends on the primer sequence and the extent of variation in specific genotype [11]. 138 loci were generated and the percentage of polymorphic loci ranged from 32.6% to 59.4% (Table 1), with

an average of 45.1%. The mean Shannon's diversity indices of all populations deviated from the maximum value (1.000) of possible diversity for a species. The mean Shannon's diversity indices ranging from 0.1399 to 0.2354 and this indicates that *N. cadamba* trees are genetically less diverse within the population examined. Further low percentage of polymorphic loci also indicates that *N. cadamba* is genetically less diverse when compared to other plant species, such as teak (*Tectonagrandis*) plus treethat has 95.5% polymorphic loci [1] and *Asparagus acutifolius* L. with 100% polymorphic loci [12].

Table 1: Estimates of genetic diversity measures in *N. cadamba* from eight selected forests in Sarawak

Forest Type	Location	n	*I*	*P* (%)
Natural forest	Bintulu	29	0.2354	59.42
	Lawas	29	0.1863	53.62
	Matang	29	0.1681	47.10
	Niah	31	0.1555	45.65
	Simunjan	30	0.1565	43.38
	Mukah Hill	28	0.1543	41.30
Planted forest	Kanowit	30	0.1399	32.61
	Song	30	0.1597	37.68

n = number of samples; I = Mean Shannon's diversity index; P = percentage of polymorphic loci

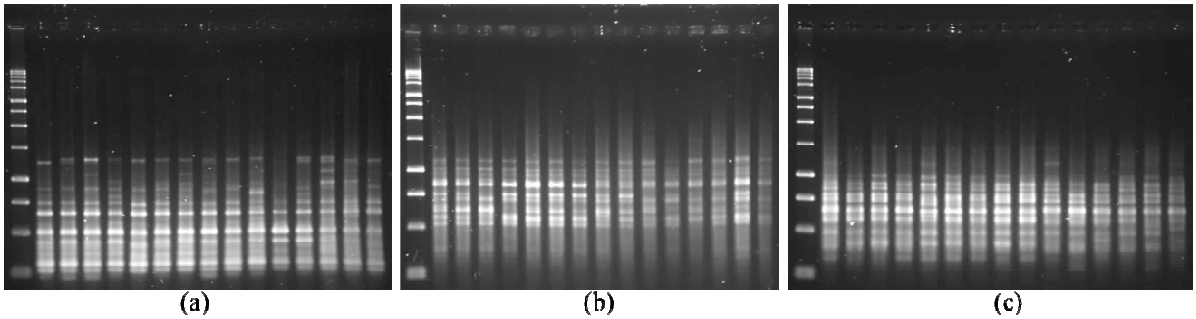

Figure 1: ISSR DNA profiles of *N. cadamba* (samples from Song, planted forest) generated by using (a) $(GTG)_6$ (5' GTGGTGGTGGTGGTGG 3'), (b) $(AC)_{10}$ (5' ACACACACACACACACACAC 3') and (c) $(AG)_{10}$ (5' AGAGAGAGAGAGAGAGAGAG 3') primers
100 bp DNA ladder was used as DNA size markers.

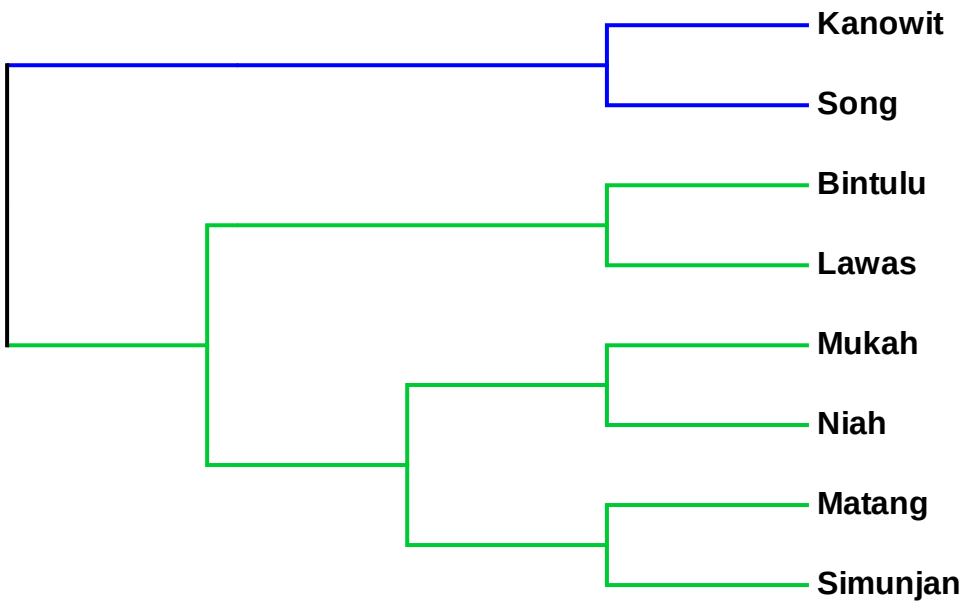

Figure 2: UPGMA dendrogram showing relationships based on Nei's genetic distance [7] of *N. cadamba* trees between six natural forest (Bintulu, Lawas, Matang, Mukah Hill, Simunjan and Niah) and two planted forests (Kanowit and Song) by using ISSR data

Figure 3: Neighbour-joining tree of 236 individuals of *N. cadamba* in eight forests performed using shared allele distance, D_{SA}[10] based on proportion of shared alleles from three ISSR markers. Each tip represents a single individual

The coefficient of population differentiation(G_{st}) of six natural forests and two planted forests was 0.2013 and 0.0871, respectively. This G_{st} value was considered low as compared to other species such as *Ceriopstagal*(Gst= 0.529) [13], *C. decandra*(0.882) [14], *Hageniaabyssinica*(Gst= 0.25) [15] and *Taxus fauna* (Gst= 0.5842) [16]. The low genetic differentiation value was also observed in other species such as *Shorealeprosula*, Gst= 0.085 [17], *Larixpotaninii*, Gst= 0.116 [18] and *Calocedrusmacrolepis*, Gst= 0.042 [19]. The low level of genetic differentiation

among populations of *N. cadamba* may be due to long-distance gene flow that has occurred over fairly large geographical areas. As explained by Hamrick *et al*. (1992)[20], long-lived woody species with large geographic ranges, outcrossing breeding systems, and wind- or animal-dispersed seeds typically display less variation among populations. Further studies are needed because there is lack of evidence regarding the reproductive biology as well as pollen and seed dispersal mechanisms of *N. cadamba*.

Among the two planted forests studied, *N. cadamba* in Kanowit's planted forest were most closely related (*I*=0.1399) compared to Song's planted forest that has a higher genetic diversitylevel (*I*=0.1597). This phenomenon might be due to the seedlings or planting materials were originated from various sources or mother trees. Meanwhile for the six natural forests, Bintulu's population has the most diverse *N. cadamba* trees (*I* = 0.2354) compared to other natural forests. On the other hand, *N. cadamba* in Mukah Hill are most closely related (*I* = 0.1555) compared to other natural forests.

Figure 2shows the genetic relationship of natural forests and planted forests in Sarawak. UPGMA dendrogram generated based on Nei's genetic distance [7]had divided natural forests and planted forests into two obvious clusters. Natural forests were grouped in one cluster meanwhile planted forests were grouped in another cluster. Same pattern was also observed when shared allele distance (D_{SA}) was used to generate neighbor-joining trees (Figure 3). 236 *N. cadamba* trees can be obviously seen to be grouped into two big clusters, which represent *N. cadamba* trees in natural forests and planted forests. This further indicates that most of the *N. cadamba* trees from different natural forests are closely related to each other. This result was also in agreement with the Shannon's diversity indices as reported earlier.

CONCLUSION

In this study, ISSR analysis was proven as a powerful tool for assessing genetic diversity of *N. cadamba* trees collected from the natural forests and planted forests in Sarawak. It clearly demonstrated that *N. cadamba* trees are closely related within its own population and its designated forest type. Thus, several specific loci can be sequenced and developed into SCAR (sequence characterized amplification region) markers which may further assist in developing the breeding and conservation strategies for the sustainable management and utilization of *N. cadamba* in future. *N. cadamba*tree with desired wood properties can then be selected for planted forest development to avoid wastage in the form of resources, economy and time.

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