

Full Length Research Paper

Genetic diversity of *Afzelia xylocarpa* (Kurz) Craib in Vietnam based on analyses of chloroplast markers and random amplified polymorphic DNA (RAPD)

Nguyen Duc Thanh^{1*}, Le Thi Bich Thuy¹ and Nguyen Hoang Nghia²

¹Plant Cell Genetics Laboratory, Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cay Giay, Hanoi, Vietnam.

²Forest Science Institute of Vietnam, Dong Ngac, Tu Liem, Hanoi, Vietnam.

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Afzelia xylocarpa (Kurz) Craib is not only an endangered species in Vietnam, but also around the world. The wood of this tree species is very valuable as it is used to construct houses and high quality furniture. Habitat loss and exploitation of *A. xylocarpa* by man have threatened the population to such an extent that the number of mature trees of this species has dwindled to quite a low quantity. Declining numbers of *A. xylocarpa* in turn causes a constriction of the gene pool. Thus, it is very important to evaluate the genetic diversity of *A. xylocarpa* (Kurz) Craib in order to conserve and sustain the surviving population of these trees. 50 samples of *A. xylocarpa* (Kurz) Craib were collected from seven locations in four provinces (Gia Lai, Dac Lac, Dong Nai and Ninh Thuan) and used to evaluate the genetic diversity of these trees based on the analysis of chloroplast 16S rRNA, non-coding regions between *trnH-trnK*, *trnD-trnT* and *psbC-trnS* chloroplast genes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and random amplified polymorphic DNA (RAPD) markers. The data obtained reveals that the 50 samples of *A. xylocarpa* (Kurz) Craib have low level of genetic diversity, as supported by the fact that the genetic similarity coefficients of the trees ranged from 49 to 100%. At the similarity level of 49%, the 50 samples were grouped into two main groups. There was no clear local specificities of the samples as the samples from same locality were not found in same group. Due to low level of genetic diversity, low numbers of trees and scattered occurrence, setting up suitable conservation strategies are urgently needed.

Key words: *Afzelia xylocarpa* (Kurz) Craib, genetic diversity, non-coding sequences, random amplified polymorphic DNA (RAPD) markers.

INTRODUCTION

Afzelia xylocarpa (Kurz) Craib (Go do or Ca Te in

Vietnamese) belongs to the family Fabaceae, subfamily Caesalpinioideae. The species is native to Vietnam, Cambodia, Laos, Myanmar, and Thailand. It is a big woody tree that can grow up to 30 m in height, with a diameter up to 2 m. In tropical forests, the trees are mostly ever green or have leaves falling. The wood of the tree is very valuable and is used to construct houses, high quality furniture, and wood carvings, while the bark of the tree is used for tanning animal hide.

A. xylocarpa (Kurz) Craib also has the potential for timber production. Furthermore, the trees fix nitrogen in

*Corresponding author. E-mail: nguyenducthanh_pcg@ibt.ac.vn. Tel: 844 37561662. Fax: 844 38363144.

Abbreviations: PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism; RAPD, random amplified polymorphic DNA; SSR, simple sequence repeat; UPGMA, un-weighted pair group method.

the soil and are thus very useful for agro-forestry and soil improvement.

High levels of exploitation by man and habitat loss have consequently decreased the number of this species of trees dramatically, and have led to the species being listed as endangered on the world list of threatened trees. In Vietnam, *A. xylocarpa* (Kurz) Craib is distributed in Kon Tum, Gia Lai, Dac Lac, and Ninh Thuan provinces. *A. xylocarpa* (Kurz) Craib can also be found in Cat Tien National Parks and Tan Phu Forest Enterprise, both in Dong Nai province. Due to the destruction of forests during the Vietnam war, coupled with an increasing population of Vietnamese people after the war, a large number of trees were cut down for living space and economic profitability, and the trees are now very hard to find.

The surviving trees are found as individuals among other species of trees in forest, but the number of mature *A. xylocarpa* in the natural forest is very low. As a result, the gene pool of the species has constricted considerably. There have not been any studies done on the genetic diversity of *A. xylocarpa* in this country, so far. Therefore, it is crucial to study the genetic diversity of the existing individuals in their native areas for the development of an effective conservation strategy.

In recent years, molecular markers have been widely used to study genetic diversity, molecular systematics and phylogenies of plant. Random amplified polymorphic DNA (RAPD) markers (Williams et al., 1990) are very popular due to their easiness of use and low cost. RAPD has been used for a variety of purposes, such as phylogenetics (Federici et al., 1998; Nicolosi et al., 2000; Ahmed et al., 2005), analyzing genetic relationships (Nicese et al., 1998; Zhang et al., 2005), analyzing species boundaries (Jimenez et al., 2005), and looking for evidence of hybridization (Gonzalez-Perez et al., 2004). The chloroplast DNA markers have also been used to study the phylogeny of plants. Chloroplast genomes are highly conserved, highly species specific, and inherited either maternally or paternally. Several chloroplast markers widely used are 16S rRNA, *rbcL*, *atpB*, *matK*, and non-coding sequences between chloroplast genes such as *trnH-trnK*, *trnD-trnT* and *psbC-trnS*. These markers have been used to study the phylogeny and systematics of Citrus (Nicolosi et al., 2000), Leguminosae and Hemerocallidaceae (Noguchi et al., 2004), Araliaceae (Plunkett et al., 2004) and Dipterocarpaceae families (Wolfe et al., 1997; Dayanandan et al., 1999).

This paper presents the results of the analysis of the genetic diversity of *A. xylocarpa* (Kurz) Craib trees based upon chloroplast and nuclear markers (RAPD). This is the first work in the country done in the application of molecular markers in the study of genetic diversity of an economically valuable tree that is highly threatened. Based on the results of this study, strategies for the conservation and development of *A. xylocarpa* (Kurz) Craib will be proposed.

MATERIALS AND METHODS

Plant samples

Leaves from 50 individual *A. xylocarpa* (Kurz) Craib trees were collected from seven localities in four provinces (Table 1).

Primers

20 RAPD primers (Set B and C, Operon Technologies Inc.), 4 chloroplast primer pairs (16SF -16SR (Tsumura et al., 1995), *trnH-trnK*, *trnD-trnT*, and *psbC-trnS* (Demesure et al., 1995) for amplification of chloroplast 16S rRNA gene and sequences (introns and spacers) between respective genes, and eight chloroplast simple sequence repeat or chloroplast microsatellite (cpSSR) primer pairs: *RCt1* to *RCt8* (Ishii and McCouch, 2000) were utilized in this study.

Restriction enzyme analysis

The monomorphic fragments of chloroplast genes, introns and spacers amplified by PCR were digested with restriction enzymes for possible generation of polymorphism in *A. xylocarpa* (Kurz) Craib samples. Three enzymes: *HinfI*, *HaeIII* and *TaqI* (Promega, Maddison, WI, USA) were used for this purpose. The digestions were carried out as recommended by the supplier.

DNA isolation

Genomic DNA of *A. xylocarpa* (Kurz) Craib was extracted from the powder of leaf samples using the modified protocol of Saghai et al. (1994). The fine powder of leaf samples was obtained by grinding liquid-nitrogen-dried leaves using a sterile mortar and pestle. In a 2 ml Eppendorf tube, 200 mg of a sample of the leaf powder was added along with an extraction buffer (preheated at 65°C) containing 4% cetyltrimethyl ammonium bromide (CTAB), 100 mM Tris-HCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 1.4 M NaCl, 5 mM ascorbic acid, 2% polyvinylpyrrolidone (PVP), 0.2% mercaptoethanol (all reagents are from Sigma Chemical). The contents of the tube were mixed well and incubated at 65°C for 60 min. After incubation, 700 µl of chloroform:isoamyl alcohol (24:1, v/v) was added to the Eppendorf tube. The mixture was then gently mixed by inversion for 5 min and later centrifuged at 10,000 g for 10 min. The supernatant was pipetted into a new 1.5 ml Eppendorf tube. DNA was precipitated by the addition of 2/3 volume of ice cold isopropanol and was centrifuged at 13,000 g for 10 min. The pellet obtained was washed twice with 250 µl of 70% ethanol, dried, and dissolved in 250 µl of Tris-EDTA (TE) buffer. DNA quality and quantity were determined in 0.8% agarose gel. Part of the obtained DNA was diluted to working concentration (20 ng/µl).

Polymerase chain reaction (PCR) amplification with RAPD primers

The 25 µl reaction mixture consisted of 2.5 µl PCR buffer, 1.5 mM MgCl₂, 200 µM of each 2'-deoxyadenosine-5'-triphosphate (dATP), 2'-deoxythymidine-5'-triphosphate (dTTP), 2'-deoxycytidine-5'-triphosphate (dCTP), and 2'-deoxyguanosine-5'-triphosphate (dGTP), 10 ng primer, 30 ng genomic DNA, and 1 unit of DNA Taq polymerase. The amplifications were carried out on a PTC-100 Thermocycler (MJ Research Inc, USA) using the following program: 94°C for 4 min, 45 cycles of 94°C for 1 min, 34°C for 1 min, 72°C for 2 min, and 72°C for 7 min to complete the reaction. The amplification products were run on 1% agarose electrophoresis gel.

Table 1. Locality, number and code of *A. xylocarpa* samples.

Locality	Province	Number of samples	Code
Cat Tien National Park	Dong Nai	10	C1 to C10
Bac Ai	Ninh Thuan	5	B1 to B5
Ngoan Muc	Ninh Thuan	5	N1 to N5
Yokdon National Park	Dac Lac	2	D1 and D2
Lac district	Dac Lac	10	L1 to L10
Eakmat	Dac Lac	8	E1 to E8
Kon Ha Nung	Gia Lai	10	K1 to K10

Table 2. Percentage of polymorphic bands of RAPD primers in 50 *A. xylocarpa* (Kurz) Craib samples.

Primer	Bands from all 50 samples	Polymorphic band	Percentage of polymorphic band (%)
OPB6	248	204	82.25
OPB10	237	188	79.32
OPB17	169	127	75.15
OPB20	122	73	59.84
OPC8	100	58	58.00
OPC9	168	127	75.60
OPC13	227	179	78.85
OPC18	242	92	38.02
OPC20	278	178	64.03
Total	1791	1226	68.45

After electrophoresis, the gel was stained with ethidium bromide and photographed by CSL MicroDoc system (Clever Scientific LTD., USA).

PCR amplification with chloroplast primers

The amplifications with chloroplast primers were performed using 25 µl reaction mixture consisting of 2.5 µl PCR buffer, 1.5 mM MgCl₂, 200 µM of each dATP, dTTP, dCTP and dGTP, 10 ng each primer, 20 ng genomic DNA, and 0.5 units of DNA Taq polymerase. The amplifications were carried out on a PTC-100 Thermocycler (MJ Research Inc, USA) using the following program: 94°C for 4 min, 40 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1.5 min, and 72°C for 10 min. The amplification products were run on 1% agarose electrophoresis gel. For chloroplast microsatellite (cpSSR) primers, the total 20 µl reaction mixture comprised 2 µl PCR buffer, 2 mM MgCl₂, 200 µM of each dATP, dTTP, dCTP and dGTP, 10 ng of each primer, 20 ng genomic DNA and 0.5 units of DNA Taq polymerase. The PCR products were resolved on 5% denaturing polyacrylamide gel. Electrophoresis was carried out at constant power (75 W) for 2 h. After electrophoresis, the gels were stained with silver nitrate. For silver staining, the gels were fixed in fix/stop solution (10% acetic acid) for 30 min. After washing the gels (3 min) with distilled water, the gels were stained in staining solution (0.2% silver nitrate, 0.05% formaldehyde) for 30 min, and then rinsed with distilled water for 10 s and transferred to pre-chilled (4 to 10°C) developer solution (3% sodium carbonate, 0.05 formaldehyde, 0.0002% sodium thiosulfate) until the bands became visible (5 to 7 min).

Data analysis

As indicated by the DNA markers used, only RAPD generated

polymorphic fragments, therefore, genetic similarity and cluster analyses were performed based on RAPD data set. For this purpose, RAPD bands were scored for each primer in each sample starting from large to small fragments. The presence and absence of a band in each sample were coded as 1 and 0, respectively. The scores were used for the creation of a data matrix to analyze genetic relationships of the individuals using NTSYS-pc program (Rohlf, 1993). Jaccard's similarity coefficients were computed and cluster analysis was studied using un-weighted pair group method (UPGMA).

RESULTS

RAPD analyses

Out of the 20 RAPD primers used, nine gave consistent PCR patterns and clear polymorphic bands across the 50 samples. Each of these nine primers generated two to six bands (fragments) of 200 to 1400 bp in size, resulting in a total of 1791 bands among 50 samples. Of them, 1226 (68.45%) polymorphic bands were recorded (Table 2). The data was used to make the matrix data set in order to analyze the genetic relationships and cluster the samples. The genetic similarity coefficients ranged from 0.395 to 1.0. The UPGMA cluster analysis showed 50 *A. xylocarpa* (Kurz) Craib samples grouped into two distinct groups (Figure 2). The two groups were genetically similar at 49%. The first group consisted of eight samples from four localities (E1, L6, and L7 from Dac Lac, K4 and K9 from Gia Lai, and N1, N2, and N3 from Ninh Thuan)



Figure 1. Sample collection localities of *A. xylocarpa* (Kurz) Craib.

with similarity coefficients ranging from 0.75 to 1.0. The second group comprised most of the samples from all the seven localities in the four provinces with similarity coefficients ranging from 0.58 to 1.0. In the first group, E1 and N2; L6, L7, and N3 were completely identical. In the second group, five pairs of the samples: D1-D2, C4-C7, C6-C9, E5-K1 and B1-K10 were completely identical. There was no clear local specificities of the samples as the samples from the same locality were not found in the same group.

RAPD markers were also analyzed for the samples from the four geographic regions. The highest similarity was obtained from samples from Dong Nai (10 samples), with the genetic similarities ranging from 0.711 (C2 to C9) to 1.00 (C4 to C7 and C6 to C9). The level of genetic similarity of the samples from Dac Lac (20 samples) ranged from 0.641 (L7 and L9) to 1.00 (L6 and L7). The samples from Gia Lai (10 samples) and Ninh Thuan (10 samples) were more divergent, for the genetic similarity was lower than that of the samples from Dong Nai and Dac Lac. The similarity values among the samples from Gia Lai and Ninh Thuan ranged from 0.422 (K5 to K9) to 0.97 (K1 to K7) and 0.348 (N3 to N5) to 0.967 (N1 to N2), respectively.

Analyses of chloroplast fragments and chloroplast SSR (cpSSR)

After running PCR with three primer pairs *trnH-trnK*,

trnD-trnT, and *psbC-trnS*, in each of the 50 samples, only one respective chloroplast fragment with the expected sizes of 1750, 1600 and 1650 bp was generated. Similar results were also obtained in the case of chloroplast 16S rRNA as only one fragment of about 1.5 kb was generated in each of the 50 samples (Figure 3a). Thus, no polymorphism was detected in the samples based on the analyses of the three chloroplast regions and the chloroplast 16S gene. The same results were obtained using eight cpSSR: each cpSSR primer pair gave similar fragments with expected sizes in all the 50 samples and no polymorphism was observed (Figure 4). For chloroplast markers, in an attempt to generate polymorphism from monomorphic fragments, three restriction enzymes *HinfI*, *TaqI* and *HaeIII* (Promega) were used. From other work (Nicolosi et al., 2000) and our test, we chose these enzymes, because they proved to be the most effective at cleaving sequences generated by the chloroplast primers utilized. They could recognize one to three restriction sites. After restriction digestion of all generated monomorphic chloroplast fragments, no polymorphism was revealed. An example of restriction digestion of chloroplast 16S rRNA fragment is as shown in Figure 3b. Thus, the analyses of chloroplast fragments and cpSSR showed that all the samples might have the same origin.

DISCUSSION

In Vietnam, *A. xylocarpa* (Kurz) Craib is distributed mainly in Kon Tum, Gia Lai, Dac Lac, Ninh Thuan and South-east provinces. Presently, *A. xylocarpa* (Kurz) Craib is mainly conserved in Nam Ca forest (Lac District, Dac Lac) and Cat Tien National Park. In other locations, *A. xylocarpa* (Kurz) Craib trees can be found as individuals among other species in forest. In this study, the samples of the *A. xylocarpa* (Kurz) Craib were collected from seven localities (populations) in 4 isolated provinces (Figure 1) from coastal (Ninh Thuan) to main land (Dong Nai), and high land areas (Dac Lac and Gia Lai). The results from RAPD analyses have reflected the natural distribution of *A. xylocarpa* (Kurz) Craib in investigated sites. RAPD analyses showed the diverseness of the samples, as most of them were genetically different and the samples from each locality did not group in the same cluster. However, the level of difference was low, in particular, the samples from Cat Tien National Park of Dong Nai province.

In addition to nuclear marker (RAPD), in this study, three types of chloroplast markers (chloroplast 16S rRNA, non-coding regions between chloroplast genes and chloroplast SSR) were used in an attempt to detect the genetic variations and possible occurrence of other *Azelia* species in the studied localities. In general, chloroplast sequences are more, specifically, conserved than those of nucleus due to the low level of substitutions in the chloroplast genome, but the levels of conservation

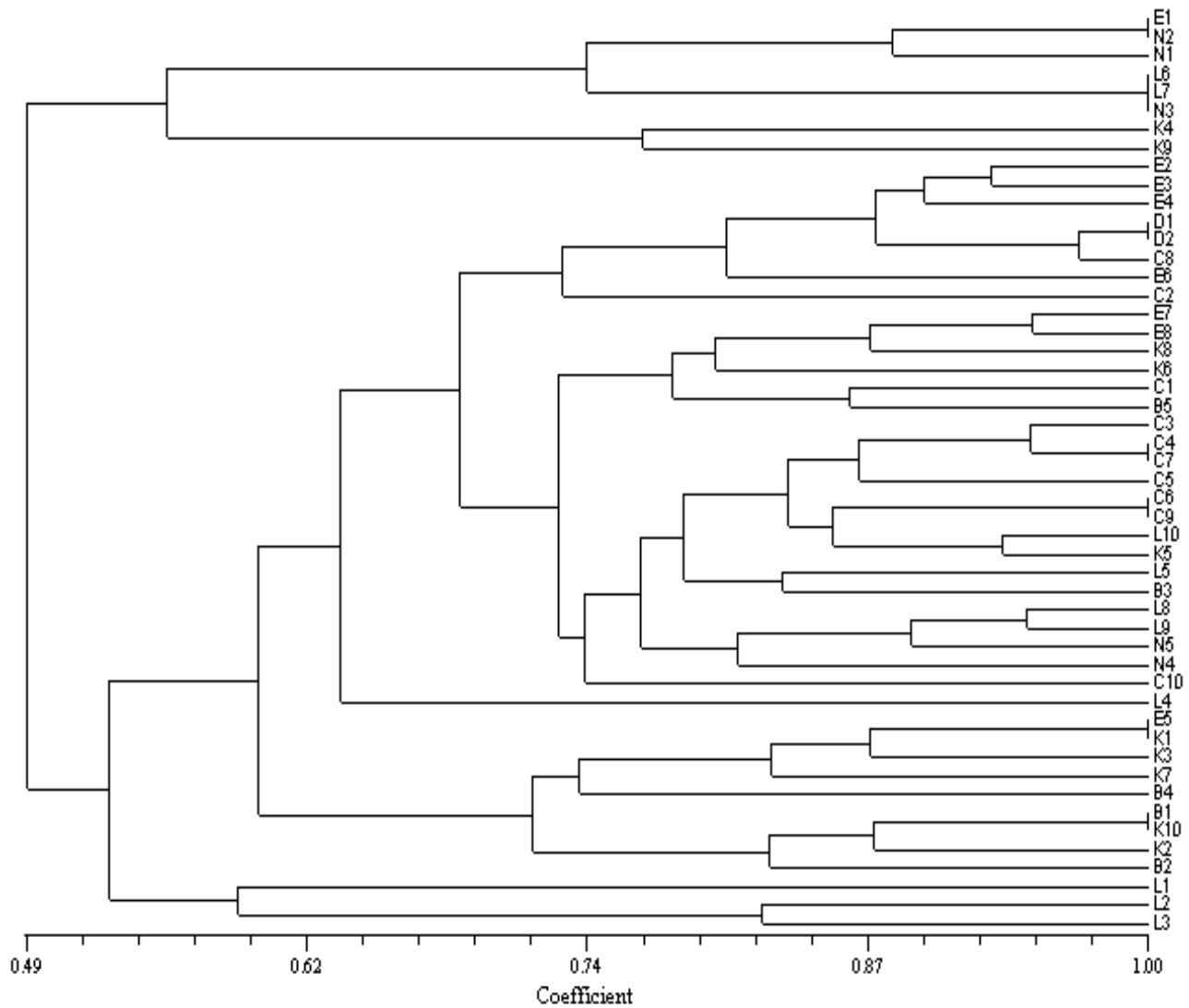


Figure 2. A dendrogram of 50 *A. xylocarpa* (Kurz) Craib samples generated by UPGMA clustering analysis using Jaccard's coefficient: B and N, samples from Ninh Thuan; C, samples from Dong Nai; K, samples from Gia Lai; D, E and L, samples from Dac Lac.

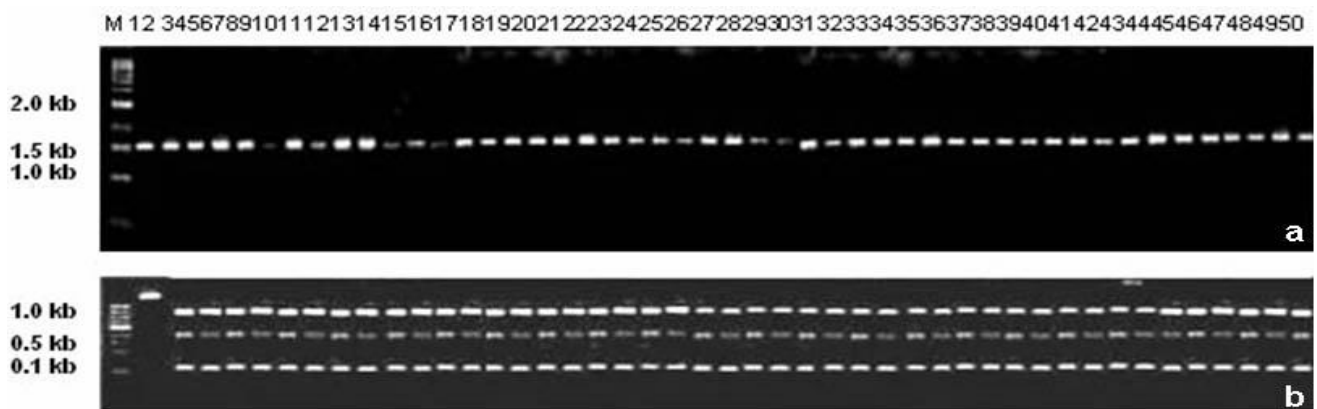


Figure 3. Amplification of chloroplast 16S gene (a) and restriction patterns (b) of chloroplast 16S gene fragments from 50 *A. xylocarpa* (Kurz) Craib samples detected by 16S/*Hinf*I primer/enzyme combination. Lanes 1 to 50, Sample numbers; M (a) 1 kb DNA marker and (b) 100 bp DNA marker.

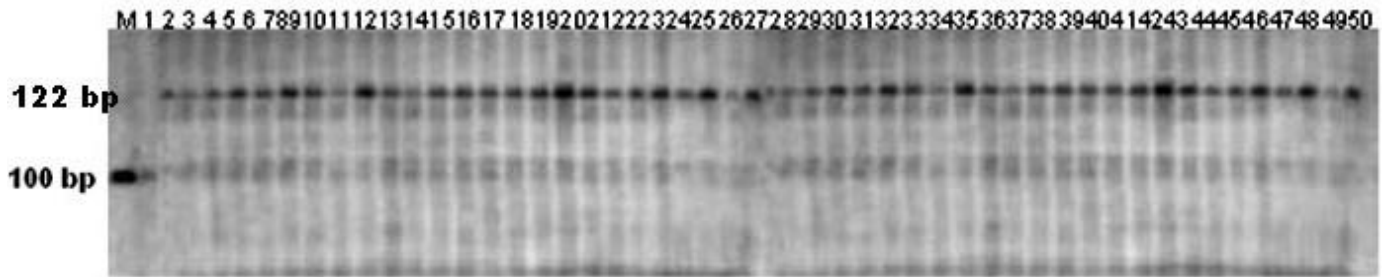


Figure 4. Amplification of chloroplast SSR region (122 bp) from 50 *A. xylocarpa* (Kurz) Craib samples using *Rct2* primer pairs. Lanes 1 to 50, sample numbers; M (100 bp DNA marker).

of different sequences are not similar. Chloroplast 16S rRNA is the most conserved. The non-coding regions between chloroplast genes and cpSSR are more variable and they can reveal the intraspecific variations. However, the studies of chloroplast microsatellites have revealed much higher levels of variation than those of RFLP analysis of PCR amplified specific chloroplast gene regions (Provan et al., 1997). The application of RFLP analysis of PCR amplified specific chloroplast gene regions has been used to study the phylogeny of plant species (Tsumura et al., 1995; Wolfe et al., 1997; Dayanandan et al., 1999; Lee and Downie, 2000, 2006; Nicolosi et al., 2000; Noguchi et al., 2004; Plunkett et al., 2004). Chloroplast SSR was used to study the population diversity of red pine and Norway spruce (Echt et al., 1998; Vendramin et al., 2000). In this study, we used similar analysis methods using chloroplast 16S gene and three non-coding regions between *trnH-trnK*, *trnD-trnT*, *psbC-trnS* genes, and eight cpSSR. The analysis using the three restriction enzymes, *HinfI*, *TaqI*, and *HaeIII*, to cleave these chloroplast fragments, showed no differences in the 50 investigated samples. In addition, no polymorphism was observed in the 50 samples after amplification using 8 cpSSR. The possible reasons for this are the conservative of chloroplast genome and the samples were from the individuals of single species (*A. xylocarpa*) in isolated localities. Therefore, the obtained data shows that there was no other *Azela* spp., but *A. xylocarpa* (Kurz) Craib occurred in the investigated localities and the samples might have the same origin. Thus, the analyses of chloroplast markers in this study have also reflected the natural occurrence of *A. xylocarpa* (Kurz) Craib in four provinces of Vietnam.

Our results indicate that all the 50 samples of *A. xylocarpa* (Kurz) Craib from the seven localities in four isolated provinces have low level of genetic diversity, illustrating the decline of gene source of this tree species. Therefore, it is recommended that suitable protection measures should be taken, because the conservation of this tree in Vietnam is urgently needed. One possible strategy could be the setup of a protected area to preserve all surviving individuals of *A. xylocarpa* (Kurz) Craib and furthermore, promote new plantings of *A.*

xylocarpa (Kurz) Craib through artificial propagation from local sources.

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