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Article

Analysis of the genetic diversity between plantation grown and natural grown *Kandelia obovata* by the inter-simple sequence repeats (ISSR) method

Janine Caynap and Katsuhiko Kondo

Abstract: Using the inter-simple sequence repeats (ISSR) method, the genetic diversities among a plantation population and two natural populations of *Kandelia obovata* Sheue, Liu & Yong were investigated. Eleven primers produced 114 band loci across a total of 60 individuals of *K. obovata* from the three populations. The results showed a relatively high genetic diversity at the species level (*P*= 76.32%, *He*=0.1725, *SI*=0.2788) making the ISSR analysis a powerful tool for assessing genetic diversity of the species. Genetic diversity in the plantation population (*P*=42.11%, *He*=0.1047, *SI*=0.1685) was lower than the two natural populations (Mean *P*=59.65%, *He*=0.1692, and *SI*=0.2652), owing to the cumulative effects of founder events, small population size and geographical isolation. The AMOVA revealed that most of the genetic variation was accounted for within populations (84%) with only 16% among the populations studied, indicating that the genetic diversity of the plantation population was fairly conserved. However, the result of the pairwise population differentiation, PHI_{PT} analysis indicated highly significant genetic differentiation between all pairs in the three populations ($P<0.01$). The dendrogram based on the genetic distances showed two major clusters, separating the natural populations from the plantation population. The highly significant genetic differentiation of the plantation population from the natural populations highlights the need to enhance its genetic diversity by continued periodical planting of significant amount of seedlings from highly diverse natural populations.

Keywords: Founder events; Genetic diversity; Geographical isolation; ISSR; *Kandelia obovata*

Introduction

The mangroves play an important role in our ecosystem. However, despite their ecological importance, mangrove forests are disappearing continuously as a direct result of human activities such as conversion to agriculture, aquaculture, tourism, urban development and overexploitation all over the world (Alongi, 2002; Giri *et al*., 2008). To counteract the destruction made on the mangrove forests, activities to conserve existing forests and rehabilitate the denuded ones by establishing mangrove plantations are now being done in various parts of the world. The upsurge of mangrove restoration efforts started around the end of the 20th century where scientific concern began to focus on the unprecedented loss of naturally occurring mangrove ecosystems around the world (Walsh *et al*., 1975) and due to the increased appreciation of their values. However, whether the genetic diversity of the species is also conserved in the process, needs to be investigated. Genetic diversity is critical for the long term survival of a species. High genetic diversity means high potential to respond to new selection environmental changes (Ge and Sun, 1999). To attain a successful conservation program, knowledge of the genetic diversity within and among populations is important (Li and Chen, 2009). One possible way to assess if a conservation program, i.e. mangrove plantations, has been successful could be by examining if the genetic diversity of the planted species was maintained.

In this study, the ISSR method was used to investigate the genetic diversity of plantation grown and natural grown mangrove species *K. obovata* Sheue, Liu & Yong, one of the major mangrove species in Japan and was formerly recognized as *K. candel* (L.) Druce (Sheue *et al*., 2003). ISSR analysis involves the polymerase chain reaction (PCR) amplification of regions between adjacent, inversely oriented microsatellites using a single simple sequence repeat (SSR)-containing primer (Zietkiewicz *et al*., 1994). The ISSR method

Laboratory of Plant Genetics and Breeding Science, Department of Agriculture, Faculty of Agriculture, Tokyo University of Agriculture, 1737 Funako, Atsugi-City, Kanagawa Prefecture 243-0034, Japan.

Table 1. Three populations of *Kandelia obovata* studied

Population code	Population location	Population type	Latitude	Longitude	Sample size
AOR	Aono River, Minami-Izu, Shizuoka, Japan	plantation	34°38'N	138°53'E	20
MIR	Minato River, Nishi-no Omote City, Tanegashima Island, Kagoshima, Japan	natural forest	30°50'N	130°57'E	20
OHR	Oh-ura River, Nakatane- cho, Tanegashima Island, Kagoshima, Japan	natural forest	30°27'N	130°57'E	20

is a useful tool in evaluating genetic diversity and has been used in several studies in mangrove species such as those belonging to *Ceriops* (Huang *et al*., 2008), *Sonneratia* (Li and Chen, 2008; 2009), *Lumnitzera racemosa* Willd. (Su *et al*., 2006), and also *Heritiera littoralis* Aiton (Jian *et al*., 2004).

The purposes of this study were: (i) to determine if there is a change in the genetic diversity of *K. obovata* individuals growing in plantation sites as compared to individuals in natural forests; (ii) to provide baseline data on genetic diversity of the species in particular areas that could be of potential use in restoration activities for this species, such as identification of germplasm sources; (iii) to evaluate the effectiveness of plantation activities in conserving the genetic diversity of the species; and (iv) to evaluate the efficiency of ISSR markers in studying this species.

Materials and Methods

Sampling areas

A plantation site studied was situated in the mouth of Aono River, Minami-Izu, Shizuoka prefecture, Japan (Table 1, AOR). This plantation was established by planting seedlings collected in Tanegashima Island in 1959 (51 years old by the year of 2010) according to Takeshita and Noguchi (1975). Two natural populations of *K. obovata* studied were located in Tanegashima Island, Kagoshima Prefecture: MIR was situated along Minato River and OHR was situated in Oh-ura River in the south (Table 1). Total of 60 individuals from the populations of *K. obovata* were collected by stratified sampling and stored at -80°C until DNA extraction.

DNA extraction and PCR amplification

Total genome DNA was isolated using the modified CTAB method (Doyle and Doyle, 1987). Three hundred to 500 mg frozen leaf samples were homogenized with liquid nitrogen and put in individual 2 ml tubes and washed once with washing buffer (0.1 M Tris-HCl pH 8.0, 2% 2-mercaptoethanol, 1% polyvinylpyrrolidone (PVP) and 0.05 M ascorbic acid). Two percent CTAB extraction buffer containing 2% 2-mercapthoethanol and 2% polyvinylpyrrolidone was then added and incubated at 60°C for at least 30 minutes. After incubation, the samples were centrifuged and the supernatant was collected. The DNA was then isolated with 500 μl 24:1 chloroform-isoamyl alcohol (CIA), precipitated with equal volume of 2-propanol, and washed with 500 μl 70% ethanol. The DNA pellet was briefly dried at room temperature, resuspended in 500 ml sterilized distilled water, added with RNase for a final concentration of 10 μg/ml and incubated for 30 minutes at 37°C. The solution was then washed with 50:50 volume of Phenol:Choroform to remove the proteins and followed by 24:1 CIA wash to remove the remaining phenol. The final precipitation was done by adding 1/10 volume of 7.5M ammonium acetate and 2.5 volumes of 99.5% ethanol, and incubated at -80°C for 15 minutes. The DNA pellet was collected by centrifuge, washed with 500 μl 70% ethanol and was completely dried before resuspended in appropriate amount of sterilized distilled water. The DNA concentration was quantified using a spectrophotometer and a working sample solution, with concentration of 100 ng/μl, was prepared and stored at -20°C. Nineteen readily available ISSR primers: $(AC)_8GG$, $(TC)_8C$, $(TG)_8G$, $(AC)_8GA$, $(GA)_8C$, $(AC)_8C$, $(AG)_8G$, $(CT)_8A$, $(TG)_8A$, $(AG)_8(CT)C$, $(AG)_8(CT)A$, $(GA)_8(CT)T$, $(GA)_8(CT)G$, $(CA)_8(AG)C$, $(AC)_8G$, $(AC)_8(CT)G$, $(ATG)_6$, $(GAA)_6$,

Table 2. ISSR primers used, sequence, annealing temperature and loci after amplification

Primers	Primer sequences	Annealing temperature (°C)	No. of loci scored	No. of polymorphic loci
ISSR 1	$(AG)_8G$	52	9	6
ISSR 2	$(AG)_8(CT)A$	52	9	7
ISSR 3	$(GA)_8(CT)T$	52	9	7
ISSR 4	$(GA)_8(CT)G$	52	4	2
ISSR 5	$(AC)_8G$	52	10	8
ISSR 6	$(AC)_8(CT)G$	52	13	11
ISSR 7	$(ATG)_6$	52	14	8
ISSR 8	$(GAA)_6$	52	13	11
ISSR 9	$(AC)_8GG$	52	11	9
ISSR 10	$(GA)_8C$	52	8	6
ISSR 11	$(AC)_8C$	52	14	12
Total			**114**	**87**

Table 3. The genetic variation statistics among sampling populations of *K. obovata*

Population	No. of polymorphic loci	*P* (%)	*Na*	*Ne*	*He*	*SI*
Plantation population						
AOR	48	42.11	1.4211±0.4959	1.1611±0.2655	0.1047±0.1525	0.1685±0.2278
Natural populations						
MIR	71	62.28	1.6228±0.4868	1.3040±0.3358	0.1869±0.1838	0.2886±0.2641
OHR	65	57.02	1.5702±0.4972	1.2315±0.2854	0.1514±0.1626	0.2419±0.2412
Mean	68	59.65	1.5965±0.4922	1.2678±0.3106	0.1692±0.1732	0.2652±0.2526
At the species level	87	76.32	1.7632±0.4270	1.2655±0.2964	0.1725±0.1632	0.2788±0.2327

P: the percentage of polymorphic loci; *Na*: observed number of alleles; *Ne*: effective number of alleles; *H*e: the mean expected heterozygosity; *SI*: Shannon's information index. See Table 1 for abbreviations of the populations.

and $(AGT)(ACG)(AGT)(TC)_7$ were initially screened and 11 of which that produced reproducible and clear fragments were selected (Table 2) and used to provide polymorphic markers for genetic diversity.

Polymerase chain reaction (PCR) amplifications were performed in 20 μl reactions, consisted of 1.25 U Taq DNA polymerase (Takara Ex Taq), 2 μl 10*x* reaction buffer containing 20 mM $MgCl_2$, 1.6 μl of dNTP mixture (2.5 mM each), 0.25 μM primer, and 100 ng template DNA. Amplification was performed using the same cycle profile as previously described by Jian *et al*. (2004). The amplification products were separated on 2% agarose gels buffered with 1*x* TAE and stained with ethidium bromide to visualize the bands. Band size was estimated using 200 bp Takara DNA ladder.

Data analysis

ISSR bands were assigned for each primer and scored as present (1) or absent (0) for each sample. The binary data matrix was analyzed by POPGENE version 1.31, assuming that the populations were in Hardy-Weinberg equilibrium. The following indices were used to quantify the amount of genetic diversity within the populations examined: the percentage of polymorphic loci (*P*), the mean expected heterozygosity (*He*) (Nei, 1973), and Shannon's information index of diversity (*SI*). Genetic diversity indices (*P*, *He*, and *SI*) were also calculated at the species level. Unbiased measures of genetic distance and genetic identity between populations (Nei, 1978) was calculated using POPGENE version 1.31 and the generated genetic distance data was used to construct a group average dendrogram using the software statistiXL version 1.1.

Analysis of molecular variance (AMOVA) was performed to calculate variance components and their significance levels within and among populations using GenAlEx version 6.1 (Peakall and Smouse, 2006).

Table 4. Nei's (1978) genetic identity (above diagonal) and genetic distance (below diagonal) among the populations studied

Population	AOR	MIR	OHR
AOR	****	0.9517	0.9551
MIR	0.0495	****	0.9770
OHR	0.0460	0.0233	****

See Table 1 for abbreviations of the populations.

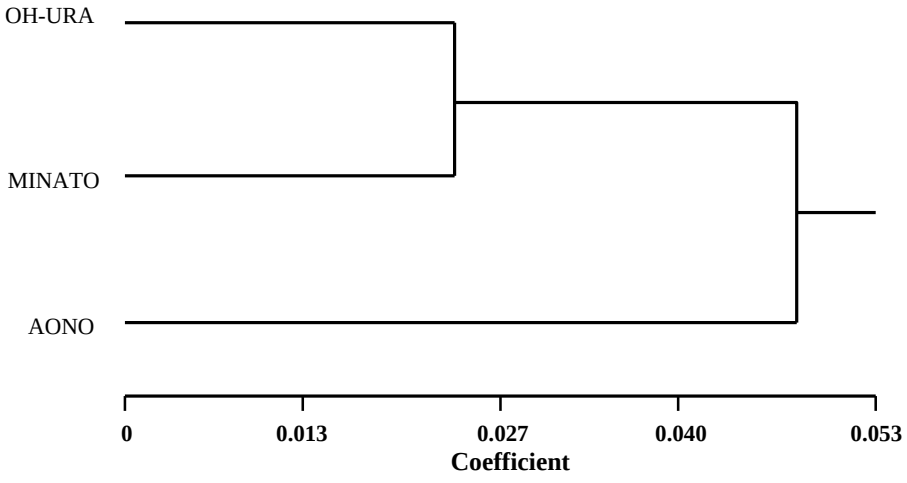

Fig. 1. Dendrogram from group average cluster analysis based on Nei's (1978) unbiased genetic distances for all population pairs.

Table 5. Summary of AMOVA

Source	df	SSD	MSD	Est. Var.	% of total variance	*P*-value	PHI_{PT}
Among Populations	2	85.133	42.567	1.685	16		
Within Populations	57	504.950	8.859	8.859	84		
Total	**59**	**590.083**		**10.544**	**100**	**< 0.01**	**0.016**

df: degrees of freedom; SS: sum of squared deviation; MS: mean of squared deviation; Est. Var.: estimated variance; PHI_{PT} : population differentiation.

Results

ISSR profile

A total of 114 reproducible band loci were detected from 60 individuals of *K. obovata* from the three populations using 11 primers (Table 2). The number of the amplified band loci per primer ranged from 4 to 14, with an average of 10.4 band loci per primer (Table 2). The size of the amplified fragments ranged from 400 to 4,000 bp. Out of the 114 bands detected, 87 (76.32%) were polymorphic (Table 2).

Genetic diversity and differentiation

At the species level, the percentage of polymorphic loci (*P*), the mean expected heterozygosity (*He*), and Shannon's information index of diversity (*SI*) were 76.32%, 0.1725, and 0.2788, respectively (Table 3). For all the population, the value of *P*, *He* and *SI* ranged from 42.11 to 62.28%, 0.1047 to 0.1869, and 0.1685 to 0.2886, respectively (Table 3). The *P*, *He* and *SI* values of MIR natural population were 62.28%, 0.1869, and 0.2886, respectively, and OHR natural population has 57.02%, 0.1514, and 0.2419. As compared to the genetic variation on the AOR plantation population, which has values of *P*=42.11%, *He*=0.1047, and *SI*=0.1685, both the natural populations showed higher genetic variation based on all the three genetic diversity indices.

The genetic distances (pairwise genetic distances shown in Table 4) between plantation population (AOR) and natural populations (MIR and OHR) were 0.0495 (AORx MIR) and 0.0460 (AORx OHR) with a mean of 0.0478. The genetic distance between the two natural populations (MIRx OHR) was 0.0233.

Cluster analysis

Based on Nei's (1978) unbiased measures of genetic distances, a dendrogram of the three populations was generated using the group average cluster analysis. The three populations were divided into two main groups: natural populations MIR and OHR were in one group, and the plantation population AOR in another group (Fig. 1).

AMOVA

Analysis of molecular variance revealed that only 16% of the total variation was accounted for among populations, while most of the variation (84%) was accounted for within populations (Table 5). Pairwise population differentiation (PHI_{PT}) analysis has values of 0.1991 (AORx MIR), 0.2103 (AORx OHR), and 0.0760 (MIRx OHR), and indicated highly significant genetic differentiation between all pairs of the three populations ($P < 0.01$) (Table 6).

Discussion

The high level of polymorphism detected (76.32%) made ISSR analysis a powerful tool for assessing genetic diversity in *K. obovata*. The result of the study of Takeuchi *et al*. (2001) on the genetic diversity of *K. candel* found in the Southwest Islands of Japan and now recognized as *K. obovata* (Sheue *et al*., 2003), revealed very low levels of genetic variations: 4.2% polymorphic loci and gene diversity of 0.012, as compared to the levels of genetic variations detected in this study: 76.3% polymorphic loci and gene diversity of 0.1725. Takeuchi *et al*. (2001) used allozyme analysis on a total of 286 samples coming from seven natural populations. However, whether the detection of genetic variations of *K. obovata* using the ISSR analysis was more effective than using allozyme analysis, cannot be concluded because of the different localities sampled. Comparing to other ISSR studies with several mangrove species (Table 7), the genetic diversity detected in this study was relatively fair. Different mangrove species were likely to exhibit varying degree if polymorphism depending on their edaphic preferences and adaptations (Lakshmi *et al*., 1997).

Kandelia obovata is usually situated in the downstream estuarine zone in the lower intertidal region (Robertson and Alongi, 1992). This species is considered a hardy species and can be easily propagated. Japan provides the northernmost and smallest habitat of the species (Takeuchi *et al*., 2001). The data revealed that the values of genetic diversity of the two natural populations in Tanegashima Island were higher than the plantation population. According to Huang *et al*. (2008), geographic range, breeding systems, and dispersal pattern are among the several factors influencing the genetic diversity of a species. Geographical isolation and habitat divergence could affect the maintenance of genetic differentiation in mangrove species (Su *et al*., 2006). The geographical location of the plantation population in Izu Peninsula, Shizuoka Prefecture was different from the two natural populations. The reason for the low genetic variation of the plantation population found along the Aono River could be

Table 6. Result of pairwise population differentiation (PHI_{PT}) analysis (PHI_{PT}) values below diagonal, probability values above diagonal)

Population	AOR	MIR	OHR
AOR	****	0.0010	0.0010
MIR	0.1991	****	0.0080
OHR	0.2103	0.0760	****

See Table 1 for abbreviations of the populations.

Table 7. Comparison of genetic diversity studies in mangrove species using the ISSR method

Species	*P* (%)	*He*	*SI*	Author (s)
Aegiceras corniculatum	16	0.039		Ge and Sun, 1999
Ceriops decandra	72	0.253	0.379	Tan *et al*., 2005
C. tagal	9	0.016		Ge and Sun, 2001
Heritiera littoralis	93	0.236	0.365	Jian *et al*., 2004
Lumnitzera racemosa	87	0.260	0.403	Su *et al*., 2006
L. littorea	75	0.240	0.357	Su *et al*., 2007
Sonneratia paracaseolaris	81	0.224	0.350	Li and Chen, 2009
K. obovata	76	0.173	0.279	Present study

P: Percentage of polymorphic loci; *He*: the mean expected heterozygosity; *SI*: Shannon's information index.

attributed to the cumulative effects of founder events, small population size and geographical isolation, lacking neighbouring populations as sources of more variation. The *K. obovata* population in Izu Peninsula, basing on its geographical location, are assumed to be subjected to colder temperature and this may also contribute to the genetic divergence of the species. The dendrogram based on the genetic distances showed two major clusters, separating the natural populations from the plantation population.

The genetic variation of *K. obovata* revealed by AMOVA was higher within populations (84%) than among populations (16%). Huang *et al*. (1994) suggested that if the genetic variation within populations in mangroves is low, it is an ecological consequence of high habitat homogenization, with physiological stress caused by unstable growing conditions. On this basis, results of this study could indicate that the genetic diversity of the plantation population along Aono River was fairly conserved. However, result of the pairwise population differentiation (PHI_{PT}) analysis indicated highly significant genetic differentiation between all pairs in the three populations ($P < 0.01$). Over time, the genetic diversity of the Aono plantation population might continue to decrease as a trend.

For mangrove conservation strategy, the genetic diversity within donor populations and their genetic similarity to the individuals existing in the plantation site should be considered for the choice of populations as sources of seeds (Hamrick and Godt, 1996). This could increase their probability of survival. As a recommendation, the genetic diversity of the individuals grown at the Aono plantation could be enhanced by continued periodical planting of significant amount of seedlings from highly diverse natural populations. Continued monitoring and assessment of mangrove plantations are essential to know the outcomes, correct or improve present situations as deemed necessary, and aid in the improvement and optimization of mangrove conservation strategies especially in terms of maintaining high genetic diversity.

Additional sampling populations, especially plantations, and more extensive sampling will provide more information to conclude the level of genetic differences between plantation grown and natural grown *K. obovata*.

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