Microsatellite analysis of genetic diversity and structure of *Bruguiera gymnorrhiza* and *Kandelia obovate*

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Abstract. Microsatellite loci were used for estimating genetic diversity and structure for three populations of *B. gymnorrhiza* and *K. obovata* (Rhizophoracea) in Okinawa, Japan. Thirty propagules of individual samples representing the population of both species were genotyped at five microsatellites. The level of observed heterozygosity (H_O) was observed for several population, overall loci, ranged 0.422-0.800 with an average 0.627 for *B. gymnorrhiza* and 0.477-0.822 with an average 0.665 for *K. obovata*, indicating both species had relatively low genetic diversity. Both species showed low levels of allelic diversity, 3-5 and 3-5 alleles per locus, respectively. Gene diversitywas also maintained within populations (H_S: 0.741 and 0.954). Additionally, an analysis of molecular variance (AMOVA) based on the immeasurable alleles model (F-statistics), for *B. gymnorrhiza* and *K. obovata* found that most of the variation resided within individuals in the total populations, i.e. 79.78 % and 69.90 % respectively, and among individuals within populations, i.e.14.30 % and 27.95 % respectively. There was little variation between populations, i.e. 5.92 % and 2.15 % for *B. gymnorrhiza* and *K. obovata*, respectively. The high-level genetic differentiation within individuals and populations both species may be due to the geographic range of the species, mating system, and environmental factors.

1 Introduction

Mangroves are typically tropical and sub-tropical coastal vegetation. In Japan, natural mangrove forests mainly distribute around Ryukyu Islands group, growing well in Okinawa,

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Iriomote Island, Ishigaki Island, and Amami Island in its northern Japanese limit on southern Kyushu [1]. The mangrove area at Okukubi River and Katabaru River, Okinawa are inhabited by four significant species, including *Bruguiera gymnorrhiza*, *Kandelia obovata, Rhizophora stylosa,* and *Lumnitera racemosa*. *B. gymnorrhiza* is denser in population, growing taller, and preferring to grow under covered conditions, while *K. obovata* and *R. stylosa* need more sunlight [2].

One of the important characterizations of mangroves Rhizophoracea is the production of viviparous offspring. Their propagules develop into seedling while they are still attached to the mother tree. In viviparous mangrove species, the embryo ruptures the pericarp, grows beyond it before dispersals such as *B. gymnorrhiza, K. obovata* and many other species of Rhizophoracea [3-4]. However, the effect of this important dispersal mechanism on the level genetic variation within and among populations is not much concerned.

In addition, the rate of gene flow through seed dispersal of a species also plays an essential role in determining population genetic structure. The restricted mangroves area in Japan, therefore the conservation and management resources have become an urgent task. Reliable information on the distribution of population genetic structure and mating system of mangrove tree species is essential to establish an active network of conservation populations [5].

Therefore, the present study was aimed to investigate population genetic diversity and structure of two mangrove species, *B. gymnorrhiza* and *K. obovata*. Information of genetic variation parameters will not only clarify the pattern of population genetic structure but also contribute to its protection, management, and utlization of mangrove tree genetic resources.

2 Materials and methods

2.1 Study site and sample collection

This study site was carried out in Okukubi River and Katabaru River, Okinawa, Japan. Thirty propagules per tree as materials for progeny arrays was used. While 30 propagules each species representing as population (Okukubi left side, Okukubi right side, and Katabaru) as shown in Fig. 1. Collected samples were kept at -80 ºC until required for DNA extraction.

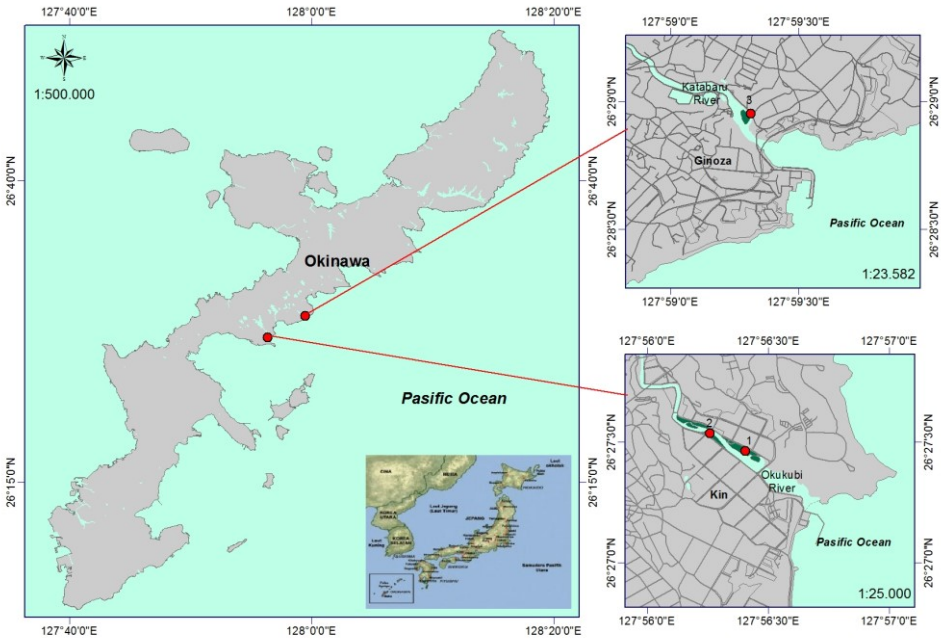

Fig. 1. Study site of Okukubi River and Katabaru River, Okinawa, Japan

2.2 DNA extraction and microsatellite analysis

Total DNA was extracted from the propagules as progeny arrays using modified CTAB procedure [6]. Five primer pairs, MSBgy025, MSBgy027, MSBgy030, MSBgy031 and MSBgy188 [7] were used in *B. gymnorrhiza* as well as five primer pairs (Kcan004, Kcan005, Kcan009, Kcan011, and Kcan034) of *K. obovata* according to Sugaya et al. [8]. PCR mixtures condition (10 µl) contained 10X Ex Taq, 1.5mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 µM of each primer (fluorescent forward and reverse), 10 ng genomic DNA and 1.0 U of Taq polymerase. PCR amplification was performed for 3 min at 94°C for, continued by 35 cycles of 1 min at 94°C, 30 s at the improved annealing temperature and 30 s 72°C, with a final extension of 5 min at 72°C using a PCR System Model PC-806 (ASTEC). The amplicons were detached using an ABI PRISMR 310 Genetic Analyzer and genotypes were analyzed by GeneScanTM and ABI PRISMTM Genotyper software version 2.0. (PE Applied Biosystems).

2.3 Data Analysis

2.3.3 Genetic diversity

Genetic diversity overall populations and all microsatellite loci were estimated as the mean number of alleles per locus (A), mean expected heterozygosity (H_E), mean observed heterozygosity (H_O) and the inbreeding coefficient (F_{IS}) using GDA, version 1.1 [9]. Genetic differentiation between populations was calculated according to Nei's gene diversity within a population (HS), gene diversity between populations (D_{ST}), the

coefficient of gene diversity (G_{ST}) [10], and calculating of allelic richness (A_R) implemented by FSTAT version 2.9.3 [11].

F-statistics (F_{IS}, F_{IT}, and F_{ST}) were computed every locus. The possibility of the *F*-statistics being more significant than zero was detected by bootstrap analysis using 1000 repeated, with a 99% confidence distance, using GDA, version 1.1 [9].

2.3.4 Genetic structure

Determination of genetic structure was done using the unbounded alleles model [12]. Analysis of molecular variance (AMOVA) [13] to quantity variance components and their importance between populations, among individuals within populations, and within populations were computed using the ARLEQUIN version 2.000 [14], in which significance levels for the overall values were decided after 1023 permutations.

To estimate gene flow, multilocus calculates the mean effective number of migrants (Nm) between population using private allele methods of Slatkin (1985) and were adjusted for sample size as provided in Barton & Slatkin [15], implemented by program GENEPOP version 3.1b [16].

3 Results and discussion

3.1 Genetic diversity

The microsatellite loci of *B. gymnorrhiza* and *K. obovata* were lowly polymorphic with 3-5 and 3-5 alleles per locus, with five microsatellite loci respectively (Table 1). In *B. gymnorrhiza*, allele sizes ranged from 200 to 257 reappearances for locus MSBgy025, from 50 to 79 repeats for MSBgy027, from 50 to 70 for locus MSBgy030, from 160 to 200 reiterations for locus MSBgy031, and from 139 to 167 replications for locus MSBgy188. At the species level, the mean observed heterozygosity (H_O), mean expected heterozygosity (H_E), mean gene diversity within the population (H_S), and mean coefficient of gene differentiation (G_{ST}) were 0.627, 0.744, 0.741, and 0.045 respectively (Table 1). At the population, the mean observed heterozygosity (H_O), mean expected heterozygosity (H_E) and mean inbreeding coefficient (F_{IS}) was 0.627, 0.926, and 0.144 respectively (Table 2).

In *K. obovata* allele sizes diverged from 390 to 480 repeats for locus Kcan004, from 350 to 400 recurrences for Kcan005, from 285 to 350 repeats for locus Kcan009, from 200 to 246 replications for locus Kcan011, and from 75 to 170 duplications for locus Kcan034. At the species level, the mean observed heterozygosity (H_O), mean expected heterozygosity (H_E), mean gene diversity within the population (H_S), and mean coefficient of gene differentiation (G_{ST}) were 0.665, 0.966, 0.954 and 0.014 respectively (Table 1). At the population, the average observed heterozygosity (H_O), average expected heterozygosity (H_E), and mean inbreeding coefficient (*F*IS) was 0.665, 0.966, and 0.298, correspondingly.

Table 1. Profile of microsatellite loci overall populations in (a) offspring *B. gymnorrhiza* and (b) offspring *K. obovata*

Locus	A	A_R	H_E	H_O	H_S	D_{ST}	G_{ST}
(a) *B. gymnorrhiza*							
MSBgy025	3	2.89	0.707	0.678	0.710	0.045	0.064
MSBgy027	5	3.56	0.790	0.433**	0.759	0.033	0.041
MSBgy030	4	3.07	0.829	0.422**	0.795	0.036	0.043
MSBgy031	4	3.50	0.791	0.800	0.740	0.053	0.067
MSBgy188	4	3.66	0.755	0.800	0.748	0.008	0.010
Mean	4	3.34	0.744	0.627	0.741	0.035	0.045

Locus	A	A_R	H_E	H_O	H_S	D_{ST}	G_{ST}
(b) *K. obovata*							
Kcan004	3	2.11	0.985	0.759	0.980	0.005	0.005
Kcan005	4	2.91	0.943	0.545**	0.933	0.013	0.014
Kcan009	5	3.27	0.976	0.822	0.953	0.023	0.023
Kcan011	4	3.90	0.971	0.722	0.969	0.005	0.005
Kcan034	5	3.47	0.952	0.477**	0.932	0.022	0.023
Mean	4.2	3..3	0.966	0.665	0.954	0.014	0.014

A: number of alleles detected, A_R: allelic richness, H_E: expected heterozygosity, H_O: observed heterozygosity, H_S: gene diversity within the population, D_{ST}: gene diversity between populations, G_{ST}: coefficient of gene differentiation. The deviation from Hardy-Weinberg equilibrium was calculated using a χ test, ** P<0.01

Table 2. Descriptive statistics over all loci for three populations of *B. gymnorrhiza* and *K. obovata*

Population	*N*	*A*	H_E	H_O	F_{IS}	Outcrossing rate
(a) *B. gymnorrhiza*						
Katabaru	30	9.2	0.754	0.673	0.108	0.805
Okukubi left	30	5.8	0.671	0.680	-0.014	1.028
Okukubi right	30	9.6	0.790	0.527	0.337*	0.495
Mean	30	8.2	0.738	0.627	0.144	0.776
(b) *K. obovata*						
Katabaru	29.6	26.6	0.931	0.704	0.241	0.612
Okukubi left	29.4	24.8	0.955	0.618	0.351*	0.480
Okukubi right	29.6	30.6	0.958	0.673	0.302	0.536
Mean	29.5	27.3	0.948	0.665	0.298	0.543

N: sample size over all loci, *A*: number of alleles detected, H_E: expected heterozygosity, H_O: observed heterozygosity, F_{IS}: inbreeding coefficient, [a] [31]. The deviation from Hardy-Weinberg equilibrium was assessed using a χ test, *P<0.1

Both species of the three populations, one involved critical departures from Hardy-Weinberg equilibrium (HWE); Which correspond to that population with substantial levels of inbreeding (F_{IS}) (Table 3). In each case, deviations from HWE were due to homozygote overabundance.

Table 3. Estimates of *F*-statistics for each locus and over all loci all populations. *F*- statistics bootstrap analysis obtains bootstrap confidence intervals *F*-statistic, using 1000 replicates and 99 % confidence interval (CI)

Locus	F_{IS}	F_{IT}	F_{ST}
(a) *B. gymnorrhiza*			
MSBgy025	-0.026	0.071	0.094
MSBgy027	0.429	0.464	0.061
MSBgy030	0.469	0.502	0.063
MSBgy031	-0.087	0.021	0.099
MSBgy188	-0.069	-0.056	0.012
Overall loci	0.153	0.209	0.066
Bootstrap CI			
Upper	0.461	0.495	0.097
Lower	-0.076	-0.031	0.031
(b) *K. obovata*			
Kcan004	0.226	0.288	0.008
Kcan005	0.415	0.427	0.021
Kcan009	0.138	0.168	0.035
Kcan011	0.232	0.238	0.007
Kcan034	0.488	0.506	0.034

Locus	F_{IS}	F_{IT}	F_{ST}
Overall loci	0.298	0.313	0.021
Bootstrap CI			
Upper	0.475	0.459	0.035
Lower	0.193	0.174	0.008

F_{IS}: inbreeding coefficient, F_{IT}: overall inbreeding coefficient, F_{ST}: fixation index

3.2 Genetic structure

To measure the degree of population distinction the infinite alleles model (F_{ST}) was used. The total value of genetic differentiation for this model resulted different values and significantly different from zero (P<0.1): F_{ST} *B. gymnorrhiza* and *K. obovata* were 0.066 and 0.021 respectively, indicates significant structure population exist within populations. The overall values of F_{IS} both were 0.153 and 0.298 respectively, suggesting little to no breeding. The overall values of F_{IT} both were 0.209 and 0.313 respectively (Table 3), indicating a moderately high correlation between alleles within individuals relative to the allelic array of the total populations.

For F_{ST}, the greater of all pairwise comparisons were significantly higher than zero (P< 0.005). Using Wright's manual [17], for F_{ST} all populations pairs in *K. obovata* and one population pair in *B. gymnorrhiza* in the limit 0.00-0.05 and two population pairs in *B. gymnorrhiza* in the range 0.05-0.15 (Table 3), suggesting moderate and little genetic diversity.

Table 4. Hierarchical analysis of molecular variance (AMOVA) of three populations of *B. gymnorrhiza* and *K. obovata*

Source of variation	d.f.	Sum of squares	Variance component		P
			Absolute	%	
B. gymnorrhiza					
Among populations	2	18.909	0.11774		<0.001
Among individuals				5.92	
Within populations	90	193.903	0.28423		<0.001
Within individuals	93	147.500	1.58602	14.30	<0.001
Total	185	360.312	1.98799	79.78	
K. obovata					
Among populations	2	12.033	0.05079		<0.001
Among individuals				2.15	
Within populations	87	258.350	0.65977		<0.001
Within individuals	90	148.500	1.65000	27.95	<0.001
Total	179	148.883	2.36056	69.90	

d.f.: degrees of freedom, P: P-value

Both species of these populations also had to estimate of the number migrants (Nm)>1, indicates some gene flow. For *B. gymnorrhiza* predicts of the number of migrants between Katabaru and Okukubi right, Katabaru and Okukubi left, Okukubi right and Okukubi left were 2.659, 1.788, and 1.189 respectively. Whereas for *K. obovata* estimates of the number of migrants between Katabaru and Okukubi right, Katabaru and Okukubi left, Okukubi right and Okukubi left were 5.128, 3,486, and 3.999 respectively. Overall populations and loci, both species the number of migrants using the private alleles method based to Barton and Slatkin [15] were 1.826 and 4.681 respectively, indicating relatively high of private alleles in these populations with the mean frequency of private alleles p(1) = 0.049 and

0.029 respectively. It is noteworthy, however, that these values of *N*m correspond to a historical average of the number of migrants per generation.

The genetic structure was also tested by analysis of molecular variance (AMOVA) using on the infinite alleles model (Table 4), both species showed that most variation was among individuals within population (14.30% and 27.35% respectively) and within individuals in the total populations (79.78% and 69.90% respectively), indicating significant genetic differentiation within individuals. There was littlevariation between populations (5.92% and 2.15% respectively).

For *B. gymnorrhiza,* the levels of genetic variation, the mean of the number of alleles per locus (*A*), and the average of gene diversity (H_E) were comparatively high with 3-5 and 0.744 respectively than those reported previously using allozymes marker [18] observed the genetic diversity values (*A*=1.10 and H_E=0.035±0.025). The levels of heterozygosity (H_O) detected for every population, overall loci (Table 3), indicate that these populations had relatively high genetic variation, these calculations of heterozygosity were much higher than those descrbed prior using allozyme to estimate H_O=0.009 [18]. Similarly, Sugaya et al. [7] also reported the genetic variation values of *B. gymnorrhiza* (*A*=2-5, H_O=0.031-0.500) using microsatellite marker.

Meanwhile, *K. obovata* also showed the high level of genetic diversity (*A*=46-73, H_E=0.966). The observed heterozygosity (H_O=0.665) was much higher than those reported previously using allozyme marker [19] found the gene diversity (H_E) and the number of alleles per locus were 0.033 and 1.11±0.04 respectively. Huang et al. [20] reported the values of genetic diversity (*A*=1.2, H_O=0.04, H_E=0.049) in *B. gymnorrhiza,* Southern China. Meanwhile, Takeuchi et al. [18] demonstrated the low level of genetic variation in *K. obovata*, Southwest of Japan (H_E=0.012±0.012). However, using microsatellite marker, Sugaya et al. [8] reported high values (*A*=3-9, H_O=0.250-0.938) in *K. obovata.* Factors such as isolation, small populations and gene flow, all of which directly affect useful population sizes, may have a significant influence on the levels of genetic diversity observed within and among populations of woody species [21]. In addition, the hierarchical analysis of molecular variance (AMOVA) of both species also showed that most of the diversity was separated among individuals within populations and within individuals in the total populations. There was little variation among populations. Differences in the breeding system may result in dissilimarity in population genetic structure. Hamrick et al. [21] pointed out that in outcrossing species, the majority of the total genetic diversity (H_E) located within populations (H_S), whereas in selfing species is more distributed among populations (F_{ST}). In this study, the genetic diversity of both species maintained within populations with the relatively high of H_S, 0.741 and 0.954 respectively (Table 4).

The comparable results were obtained for other mangroves tree species. Partitioning of variation was detected within the populations of *B. gymnorrhiza* (70%), and for *B. sexangula* 60% of the difference was observed among populations [22]. Jian et al. [23] reported the variation within populations of *Heritiera littoralis* from Australia and China and within China were 46.9 % and 72.2 respectively. Basyuni et al. [6] observed in *Rhizophora mangle* from Ecuador that the variation was partitioned among individuals within populations (33.3 %) and among individuals (66.6 %) in the total populations. Meanwhile, the genetic variation of *Sonneratia alba* in China was maintained within populations, 79.39 [24].

Also, assessment of genetic variation of population levels throughout the worldwide range of *A. marina* showed that most variation was found between populations (41-71%) and within individuals in the total populations (31-49%) [25]. Hamrick et al. [21] reported that studies on genetic diversity within populations of woody species showed that a high level of genetic diversity mostly resides within populations.

The discrete subpopulation model indicated little and moderate levels of genetic differentiation using Wright's interpretive guidelines [17] (Table 6). The extent and the pattern of genetic diversity in forest trees are strongly regulated by their mating system and gene flow [26]. Indirect estimates of gene flow using private alleles resulted relatively high for overall population of both species (*N*m=1.826 and 4.681, respectively). If *N*m is less than 1, then changes in allele frequencies resulting from a genetic drift of neutral alleles can occur. Such changes are not likely if *N*m is greater than 1 [27]. Moreover, viviparous seed dispersal is potentially a much greater source of gene flow in mangroves [20].

As measured by electrophoretically distinguishable alleles, which are often considered to be neutral variations, the value for *N*m in a range of forest tree species indicates fairly high levels (> 1) of gene flow. This is to be expected because most trees have high outcrossing rates, and there is a definite association between outcrossing rate and the level of gene flow [28]. The high value of *N*m for both species is comparable to estimates reported for other mangroves tree species, such as in *R. mangle* in Pacific coast, *N*m=3.174 [29] and *R. mangle* in Ecuador, *Nm*=3.778 [6].

Gene flow switch of gametes or genes among distributed trees, and it is reciprocally correlated to population adjustment. It occurs through the forward motion of pollen and seed, which can be incoherent by a broad varied of non-living and living mechanisms. Understanding how populations are dispread, preserved, or restricted by gene flow is indispensable for control precise tree populations in their primary environment [30].

The structure of genetic diversity within and among populations of species is an inherent feature of the evolution of mangrove forest, that may be correlated to evolutionarily oxidosqualene gene and salt tolerance gene [31-32], and must, therefore, be considered in developing any conservation strategy. Our results suggested supporting the conservations efforts of the mangrove ecosystem in Okinawa. The protection implies of the available data of these studies on genetic variation and structure are that, in the conditon of outcrossing species, some populations in Okinawan mangroves, especially *B. gymnorrhiza* and *K. obovata* may sustain much of the genetic diversity.

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