Microsatellite Analysis on Genetic Variation in Two Populations of Red Mangrove *Rhizophora Mangle* L. (Rhizophoraceae) and Its Implication to Conservation

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Abstract. Two populations of red mangrove *Rhizophora mangle* L. (Rhizophoraceae) in Ecuador were examined using five microsatellite primers. Microsatellite loci were low polymorphic with 2-5 alleles per locus. The level observed heterozygosity detected overall loci population, ranged from 0.189 to 0.405, indicating that these populations had little or no genetic variation. Furthermore, genetic variation was maintained within population (GST=0.012). Both populations showed high level of inbreeding (FIS=0.695, FST=0.024), suggested that mangrove species behave as colonizing species. The genetic structure was tested by analysis of molecular variance (AMOVA) using the infinite alleles model (F-statistics), found that most variation was among individuals within population (33.3 %) and within individuals in the total populations (66.6 %), whereas there was little variation between populations (0.1 %). An understanding of genetic variation of *R. mangle* suggested the importance of conservation this species to increase the number of populations.

1. Introduction

Mangroves are halophytes that are defined ecologically by their location in upper inter-tidal zones of tropical and sub-tropical climates and physiologically by their ability to withstand high concentrations of salt or low levels of soil aeration [1]. Mangroves are one of the most threatened ecosystems all over the world today due to direct and anthropogenic indirect degradation [2], and have resulted in great loss of genetic diversity in the mangrove ecosystem [3]. Conservation of mangrove including genetic resources implicates not only to protect the coastal areas and communities from seawater intrusion and potential changes in sea level rise but also to ensure the availability of resources for future use through adaptation to changing environments.

Information on genetic diversity of mangrove species is very important in planning for conservation of genetic resources, afforestation and tree improvement program [4]. The genetic variation of a species can be assessed by molecular markers, which are important tools for estimating genetic diversity, population genetic structure, gene flow, matting system or reproductive dynamics. These information can provide guidelines for ecological management, afforestation, and reforestation [3].
The red mangrove trees of Rhizophora mangle L. (Rhizophoracea) show a variable height and diameter, with a light gray external cortex sometimes smooth other rough with deep fissures. R. mangle is a perennial whose habitat is restricted to intertidal zones and coastal lagoons. Although several studies on population genetic R. mangle have been reported [5-7], the extent and patterns of genetic diversity in this mangrove species especially from the Pacific Coast remain obscure. Genetic diversity is a critical for adaptation to environmental changes and for long-term survival of a species. The genetic diversity has to be conserved before completely restoring the ecological role that has long been lost due to the mangrove ecosystem deforestation [4]. The objective of the present study was to assess the genetic variation within and between natural populations of R. mangle distributed in Guayaquil and Esmeraldas of Ecuadorian mangroves, using the microsatellite loci selected based on the previous report [8] to acquire useful genetic information to support mangrove forest conservation.

2. Materials and method

2.1. Sample collection
A total of 54 individuals of R. mangle leaves were collected from two natural populations in Ecuador: Guayaquil (23 samples) and Esmeraldas (31 samples). The plant tissue was dehydrated in silica gel and then stored at -80 °C until needed for DNA extraction.

2.2. DNA isolation
Total genomic DNA was extracted from leaf tissues using modified cetyl trimethyl ammonium bromide (CTAB) procedure [9]. The quality of the DNA was evaluated using 1% agarose gels and then quantified by UV-Spectrophotometer (Shimadzu, Kyoto, Japan). The material genetics were stored at -20 ºC.

2.3. Microsatellite analysis
Five primer pairs (RM6, RM11, RM21, RM38, and RM41) used in this study are designed for R. mangle as previously described [8]. PCR reaction mixtures (10 µl) contained 10X Ex Taq Buffer, 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.1 µM of fluorescent-labeled forward primer (FAM, NED, and HEX) and 0.1 µM reverse primer, 10 ng genomic DNA and 1.0 U of Taq polymerase (Takara Shuzo, Kyoto, Japan). Analysis of microsatellite loci was carried out after PCR optimization. PCR amplification was carried out for 3 min at 94 ºC, followed by 35 cycles of 1 min at 94 ºC, 30 s at 51-54 ºC and 30 s at 72 ºC, with final extension of 5 min at 72 ºC using a PCR System Model PC-806 (ASTEC). The amplification products were separated using an ABI PRISM 310 Genetic Analyzer and genotypes were determined by GeneScan™ and ABI PRISMTM Genotyper software version 2.0 (Applied Biosystems).

2.4. Data analysis
Genetic variation overall populations and all microsatellite loci were calculated using Genetic Data Analysis ver. 1.0 [10] as mean number of alleles per locus (A), mean expected heterozygosity (HE), mean observed heterozygosity (HO) and F-statistics (FIS, FIT, and FST) were estimated each locus. The probability of the F-statistics being greater than zero was determined by bootstrap analysis using 1000 replicates, with a 99% confidence interval. Genetic differentiation between populations was estimated according to Nei’s coefficient of gene diversity (GST) [11]. The outcrossing rate was calculated as \((1 - F_{IS})/(1 + F_{IS})\).

Assessment of genetic structure was performed using the infinite alleles model. Analysis of molecular variance (AMOVA) was used to measure variance components and their significance between Guayaquil population and Esmeraldas population, among individuals within populations, and within populations were calculated using the Arlequin version 3.0 [12]. Tests of significance for each value were determined after 1023 permutations.

3. Results and Discussions
The results will be discussed in three subsections; they are genetic variation, genetic structure and conservation consideration.

3.1. Genetic variation

The five microsatellite loci of *R. mangle* were low polymorphic with 2-5 alleles per locus. Locus RM6 had 5 alleles, locus RM11 had 3 alleles, locus RM21 had 5 alleles, locus RM38 had 3 alleles, and locus RM41 had 2 alleles. The observed heterozygosity (*H*<sub>O</sub>) for each locus ranged from 0.189 to 0.405 with an average 0.284 (Table 1). At the population level, mean expected heterozygosity (*H*<sub>E</sub>), and coefficient of gene differentiation (*G*<sub>ST</sub>) were 0.938 and 0.012 respectively (Table 1). The *H*<sub>O</sub> were much lower than expected from Hardy-Weinberg expectations (*H*<sub>E</sub>), indicating the presence of inbreeding in all populations (Table 1).

Very low allelic diversity (2-5 alleles per locus) was observed in this study supported previous reports on microsatellite analysis from *R. mangle*. It was found 2 to 4 alleles per locus in *R. mangle* along the northwestern coast of Mexico [7], while 3-8 alleles found on the Colombian Pacific coast [5]. Also [8] reported 2-7 alleles per locus in *R. mangle* from two natural populations of the Colombian Pacific Coast. [6] found 2-6 alleles in *R. mangle* from Brazilian coast. Twenty-three individuals of *R. mangle* from the Pacific coast of Costa Rica showed 2-6 alleles [13]. Therefore, very low allelic diversity was observed for *R. mangle* in Columbian, Costa Rican, Brazilian and Mexican coast, parallel with finding low alleles per locus from the populations at the pacific coast of Ecuador. Mangrove tree species are more likely to be monomorphic than other tree species with mixed-mating systems [4].

| Locus | N  | A  | *H*<sub>E</sub> | *H*<sub>O</sub> | *G*<sub>ST</sub> | Outcrossing rate |
|-------|----|----|----------------|----------------|-----------------|------------------|
| RM6   | 54 | 5  | 0.979          | 0.405**        | 0.008           | 0.263            |
| RM11  | 54 | 3  | 0.894          | 0.189**        | 0.009           | 0.119            |
| RM21  | 49 | 5  | 0.968          | 0.375**        | 0.003           | 0.251            |
| RM38  | 54 | 3  | 0.914          | 0.206**        | 0.040           | 0.115            |
| RM41  | 54 | 2  | 0.923          | 0.243**        | 0.001           | 0.151            |
| Mean  | 53 | 3.6| 0.936          | 0.284          | 0.012           | 0.180            |

N: number of individuals, A: number of alleles detected, *H*<sub>O</sub>: observed heterozygosity, *H*<sub>E</sub>: expected heterozygosity, *G*<sub>ST</sub>: coefficient of gene diversity

** Significant from Hardy-Weinberg equilibrium (*P*<0.01)

Both populations showed high level of inbreeding (*F*<sub>IS</sub>=0.695, *F*<sub>ST</sub>=0.024) as shown in Table 2, this value was paralleled with the relatively low of outcrossing rate (0.177) (Table 1). The significant deviations from Hardy-Weinberg equilibrium were found at all loci with positive FIS values (Table 2). Similarly, the significant heterozygote deficit was distributed at all loci, except locus RM21 of Guayaquil population (data not shown). The deviation might have resulted from the bias due to miss-amplification of loci caused by null alleles were evidently found in two loci of RM6 and RM38 of Guayaquil population and in locus RM38 of Esmeraldas population, leading to an apparent excess of homozygosity (data not shown). Indirect estimates of gene flow using private alleles between populations showed relatively high value (*Nm*=3.778). It is noteworthy that the value of *Nm* corresponds to a historical average of the number of migrants per generation. Interestingly, distribution of alleles overall loci was dominated by private alleles (data not shown). This result was parallel with relatively high of gene flow.
Table 2. Estimation of F-statistics for each locus and over all loci all populations

| Locus | $F_{IS}$ | $F_{IT}$ | $F_{ST}$ |
|-------|---------|---------|---------|
| RM6   | 0.582   | 0.588   | 0.015   |
| RM11  | 0.790   | 0.795   | 0.019   |
| RM21  | 0.600   | 0.602   | 0.005   |
| RM38  | 0.786   | 0.803   | 0.079   |
| RM41  | 0.739   | 0.74    | 0.001   |
| Overall loci | 0.695 | 0.703 | 0.024 |

Bootstrap CI

|        | Upper | Lower |
|--------|-------|-------|
| $F_{IS}$ | 0.789 | 0.589 |
| $F_{IT}$ | 0.801 | 0.594 |
| $F_{ST}$ | 0.067 | 0.003 |

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

3.2. Genetic structure of R. mangle

The genetic structure was tested by analysis of molecular variance (AMOVA) using on the infinite alleles model (F-statistics) was displayed in Table 3, found that most variation was among individuals within population (33.3 %) and within individuals in the total populations (66.6 %), whereas there was little/no variation between populations (0.1 %). All components of molecular variance were highly significant (P < 0.001) (Table 3). The genetic population showed that most of the variation was partitioned among individuals within populations (33.3 %) and within individuals (66.6 %) in the total populations. Our current studies well agree with previous studies on genetic variation of woody species, showing that a high level of genetic variation mostly resides within the populations [4].

Table 3. Summary of analysis of molecular variance (AMOVA) of two populations of R. mangle

| Source of variation | Df | Sum of squares | Variance components | % Variation | P      |
|---------------------|----|----------------|---------------------|-------------|--------|
| Between populations | 1  | 0.65           | 0.0003              | 0.10        | <0.001 |
| Among individuals   |    |                |                     |             |        |
| within populations  | 52 | 34.68          | 0.1668              | 33.30       | <0.001 |
| Within individuals  | 54 | 18.00          | 0.3333              | 66.60       | <0.001 |

P, level of significance for the distribution of variation

It has been reported [5] that the population structure of R. mangle in the Colombian Pacific, was detected at the within-population level (94.62%). Similarly, 54% of 10 populations of R. mangle at the northwestern Mexican coast were resided within individuals [7]. Additionally, 47.74% of genetic structure of R. mangle in Brazillian Coast was found also within populations [6]. Our present studies therefore well agreed with previous reports on genetic structure of R. mangle. The present study found little genetic variation between populations. The low levels of genetic variation between populations in our study may due to anthropogenic activities; these study areas of both populations have been converted into shrimp farms, agriculture and cattle fields, salt extraction ponds and settlement areas. The low genetic variation of both populations in R. mangle may be subjected to vulnerable to environmental changes [14]. Furthermore, coastal dwellers in Ecuador has been reported to harvest large quantities of mangrove wood for construction, piles, fuel wood, and conversion to charcoal [15].

3.3. Conservation consideration

Mangroves are threatened all over the world today due to direct and indirect exploitation. In Ecuador, mangroves are degraded by logging and conversion to shrimp pond for the export market [15]. Conservation of mangrove genetic resources not only to prevent extinction but also to ensure the
availability of resources for future use through adaptation to environmental changes [2]. The population genetic structure revealed here in R. mangle has clear conservation and management implications. One of the suggestions for mangrove restoration and rehabilitation is to choose genetically superior and to introduce them into target sites.

4. Conclusions

The low genetic structure of R. mangle found in this study is therefore very noteworthy in planning for its long-term conservation [4]. An understanding of the low genetic variation of R. mangle in Ecuador suggested the importance of conservation this species and management to increase the number of populations. Conservation of genetic diversity is very crucial to secure genetic material to be used in present and future of this mangrove tree species.

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