

Full Length Research Paper

# Genetic variation of mangrove species *Avicennia marina* in Iran revealed by microsatellite markers

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**Mangroves play an essential role in ecosystem dynamics but are reported to be regressing as human pressure increases on coastal zones. In order to ensure conservation of mangroves, genetic diversity in remaining population must be explored. Since *Avicennia marina* is an environmentally susceptible species, such studies including examination of its genetic variation is done in a worldwide range. During the present study the level of genetic variation of mangrove trees (*A. marina*) in three coastlines of Bushehr province (Southwest regions of Iran) was examined using microsatellite markers. Three microsatellite loci which were applied in the last large-scale study, detected high levels of allelic diversity here (14 alleles in total), essential for an accurate estimation of population genetic parameters. The levels of heterozygosity detected for each population, over all loci, ranged from 0.451 to 0.667 with an average of 0.589, indicating relatively appropriate level of genetic variation. The expected heterozygosity was larger than the observed heterozygosity leading to positive inbreeding coefficients in all three populations. Highly significant departures from Hardy-Weinberg Equilibrium were detected in populations. Reduced level of genetic variation was found in the central population indicating strong genetic structure among the other populations with larger area and less exploitation.**

**Key words:** *Avicennia marina*, genetic variation, Iran, microsatellite, population.

## INTRODUCTION

Mangrove forests are a feature of the intertidal zone of the tropical and subtropical coastlines of the world. Mangroves comprise some 80 higher plant species in several taxonomic groups (Saenger, 1998) and 70 species of true mangrove has been reported, as well (Kitamura et al., 1997). Mangrove forests all over the world are heavily exploited for wood and fishpond operations, as well as other activities. The exploitation of mangroves has resulted in the loss of genetic diversity in mangrove ecosystems (Maguire et al., 2000b), as well as the loss of valuable wood resources. To overcome these losses, conservation and sustainable management of mangrove reserves is, thus, a major priority in the coastal areas of

many countries. Population genetic studies of mangroves are, therefore, aimed at providing the information needed for afforestation, domestication and breeding programmes, and for the conservation of genetic resources. A major requirement for natural mangrove populations is the development of efficient strategies for the *in situ* conservation of genetic resources; in contrast, the goals for breeding programmes are the maximum capture of desirable genetic diversity during domestication and the production of high quality genetic seed orchards. To date, the extent and patterns of genetic diversity in natural mangrove populations in the entire worldwide range are largely known (Maguire et al., 2000b), but informative data on mangrove forests of Iran is lacking.

Mangroves form the dominant intertidal ecosystems throughout the tropical regions of the world and range from Japan (Northern limit) to Victoria, Australia (Southern limit). The total area of mangroves worldwide

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is estimated at about 182 000 Km<sup>2</sup> (Dodd et al., 1998). However, due to increasing human pressure in coastal zones, accompanied by the conversion of mangrove forests to cultivated areas, non sustainable harvesting and pollution, those ecosystems are seriously affected (Dodd et al., 1998). So, the conservation and sustainable management of mangroves is thus a major priority in coastal areas of many countries (Maguire et al., 2000b).

Mangrove forests in Iran are spread as the large and small interrupted communities with the area over 15000 ha (Safiari, 2002). The settlement area of the forests starts from eastern zone of Oman Sea in Goatr Gulf and complete in Persian Gulf westerly. The mentioned forests including only two tree species of *Avicennia marina* (with the most parts) and *Rhizophora macronata* have been studied for oil pollution impacts and some primary ecological subjects on their growth, existence, etc; but there are no researches on their genetics.

Because *A. marina* (Forsk.) Vierh can grow and reproduce across a wide range of climatic, saline, and tidal conditions, it is the most widely distributed and thus most important of all mangrove tree species. The structure of *A. marina* stands varies from gnarled shrubbery on dry coastlines and coral atolls, to closed estuarine forests up to 40 m tall within larger systems of wet coastal tropical regions (Duke et al., 1998).

Microsatellites are now being described in many plant species. Microsatellites have been reported in several forest tree species such as *Pinus* spp. (Smith and Devey 1994; Kostia et al., 1995; Echt et al., 1996), *Quercus* spp. (Dow et al., 1995), *Picea* spp. (van de Ven, 1996), *Pithecellobium* spp. (Chase et al., 1996), *Swietenia* spp. (White and Powell, 1997), and *Eucalyptus* spp. (Brondani et al., 1998). All these studies, however, resulted in the development of only a small number of markers; typically less than ten. Microsatellite markers for *Avicennia alba* were obtained by screening a partial genomic library enriched for microsatellite dinucleotide repeats, and among 20 primer pairs defined, six amplified polymorphic microsatellites with a satisfying level of variability found (Teixeira et al., 2003). Six polymorphic microsatellites DNA markers in *A. marina* (Forsk.) Vierh are determined in Maguire (et al., 2000a) explorations.

The objectives of the study described here were to determine the state of genetic variation of different populations, and to discuss their potential for application in the conservation of mangrove genetic resources, population genetics and plant breeding.

## MATERIALS AND METHODS

### Site description

Disjunctive populations of *A. marina* in three coastlines of Bushehr province were selected for investigation; namely the Bordekhoon, Dayyer and Asaluyeh located in 25 to 27° 50' north latitude and 55° 10' to 65° east longitude. The coastlines are separated from

each other by varying distances (Bordekhoon to Dayyer (60 km), Dayyer to Asaluyeh (120 km) and Bordekhoon to Asaluyeh (100 km). There are ocean-borne propagules accompanied by insect pollination likely to be the effective means of gene flow. The three shorelines varied substantially in area size, salinity, pollution, exploitation and proximity to oil pollution center. Therefore, it was assumed that they would be subjected to individual selective pressures, in addition to common pressures such as maritime conditions. The three shorelines are located within 200 nautical miles along the coast, and thus gene exchange between them is possible according to the findings of Duke et al. (1998).

### Plant material and DNA isolation

A total of 69 individuals, representing three natural populations, were sampled over Bushehr province shorelines as a transect method along east-west direction. Leaf material from each population was collected. From each of the shorelines, 21 - 25 trees were sampled at random. Leaf material was stored at -80°C for later analysis. Total genomic DNA was isolated from leaf tissue using a modified CTAB method by Maguire et al. (1994).

### Microsatellite analysis

Primer sequences for the three microsatellite loci used in this study are those applied in worldwide range study of *A. marina* presented in Table 1. There is also a comparative possibility to look up worldwide range of mangroves. Thus, markers of M3, M40 and M47 amplified the dinucleotide repeat sequences of (TG)<sub>15</sub>, (AG)<sub>32</sub> and (CA)<sub>13</sub>, respectively. Polymerase chain reaction (PCR) amplification conditions were: 1x reaction buffer (10 mm Tris-HCl, 50 mm KCl, pH 8.3 and 1.5 mm MgCl<sub>2</sub>, Roche), 200 mm each dNTP, 0.2 mm each primer, 1 unit Taq DNA polymerase (Roche) and 10 - 50 ng genomic DNA, in a total volume of 25 mL. After an initial heating step at 94°C for 3 min, samples were incubated for 30 cycles of 94°C for 30 s, 55°C for 35 s, and 72°C for 1 min. Reactions were completed by incubating at 72°C for 5 min and held at 4°C. Cycling conditions were performed using a Corbet Research (CR) Cycler. PCR products were separated in 8% non-denaturing polyacrylamide gels and visualized by ethidium bromide (a modified staining method by Giannattasio et al., 1997). The study used this method for the first time in a plant study (Table 1).

### Data analysis

Number of alleles, heterozygosity levels, Hardy-Weinberg Equilibrium and F-statistics were computed using the program popgene 32. Nei (similarity) coefficient and UPGMA program were responsible for clustering method.

## RESULTS

### Genetic diversity

The 69 individuals, representing three natural populations of *A. marina* produced a total of 14 alleles with three microsatellite loci. That is the first observation of the alleles in Iran. Locus M3 had 5 alleles, locus M40 had 5 alleles and locus M47 had 4 alleles (Table 2). The observed heterozygosity (H<sub>o</sub>) for each locus ranged from 0.348 to 0.787 (Table 2).

The total number of allele per population for all loci

**Table 1.** Characterization of the three microsatellite loci identified in *Avicennia marina*: the name, primer sequence, motif, TM and size of cloned allele.

Locus	Primer sequence (5' to 3')	Sequence	Tm (°C)	Size of cloned allele (bp)
M3	F: GGTTCTGCAAGTATGTCAACACCCTC R: ACCTCGATTCTCCCGAATGC	(TG) <sub>15</sub>	60	182
m40	F: CCCATAGATGACGGCAATCTTATGATCC R: ACCATCCAAAATAAAATAAATCTCCCTCCC	(AG) <sub>32</sub>	60	161
M47	F: TGACACCAAGGGAAATCAACATGCC R: GAACCTAGCGACCAATAGATCATCTCTGG	(CA) <sub>13</sub>	60	172

**Table 2.** Descriptive statistics for three microsatellite loci studied overall populations. A, total number of alleles; H<sub>O</sub>, observed proportion of heterozygosity; H<sub>E</sub>, expected proportion of heterozygosity.

Locus	A	H <sub>O</sub>	H <sub>E</sub>
M3	5	0.630	0.778
M40	5	0.348	0.692
M47	4	0.787	0.753

**Table 3.** Descriptive statistics overall loci for each population of *Avicennia marina*. N, number of individuals; A, number of alleles; Ave, average number of alleles; H<sub>O</sub>, observed

Population	N	A	Ave	H <sub>O</sub>	H <sub>E</sub>
Bordekhoon	23	14	4.66	0.6502	0.761
Dayyer	21	11	3.6	0.4514	0.674
Asaluyeh	25	14	4.66	0.6667	0.742

ranged from 11 - 14. The average number of allele per population ranged from 3.6 to 4.66 (Table 3). For each population, overall loci, the expected heterozygosity (H<sub>E</sub>) was generally higher than the observed heterozygosity (H<sub>O</sub>), leading to positive inbreeding coefficients (F<sub>IS</sub>). For each population observed heterozygosity ranged from 0.674 to 0.761 with an average heterozygosity of 0.725 (Table 3); overall populations and loci, F-statistic analysis showed low levels of F<sub>IS</sub>, F<sub>IT</sub> and F<sub>ST</sub>; 0.161, 0.198 and 0.044, respectively (Table 4).

### Genetic structure

No significance linkage disequilibrium was detected between different genotypes at each of the different microsatellite loci. Hardy-Weinberg Equilibrium (HWE) departures were measured using F-statistics, and the departures from zero were tested. All of populations had significant departures from HWE, which correspond to their significant levels of inbreeding. In each case, departures from HWE were due to homozygote excess. As it is shown in Table 5, two populations of Asaloooyeh and Bordekhoon have most genetic identity with the least genetic distance (having most geographic distance, how-

**Table 4.** F-Statistics analysis estimates the parameters FIS, FIT and FST for each locus and over all loci over all populations.

Locus	Repeat unit	F <sub>IS</sub>	F <sub>IT</sub>	F <sub>ST</sub>
M3	(TG) <sub>15</sub>	0.1343	0.1653	0.0359
M40	(AG) <sub>32</sub>	0.4613	0.4934	0.0595
M47	(CA) <sub>13</sub>	-0.0826	-0.0405	0.0389
Overall		0.1605	0.1977	0.0443

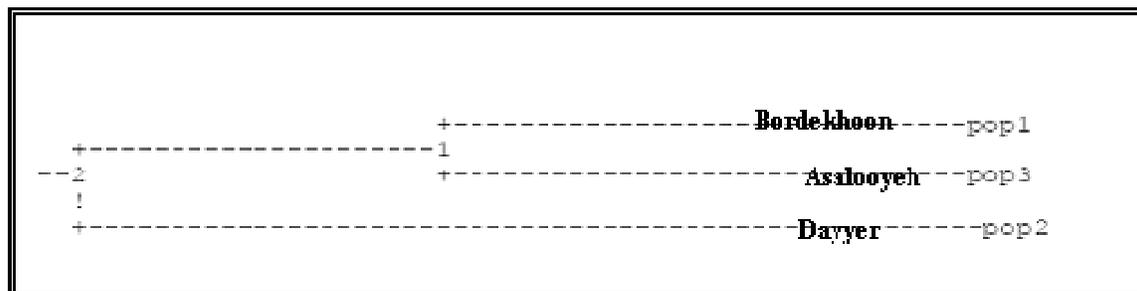
ever) indicates that the amount of genetic variation of a population would be increased as increasing the area (cluster model shown in Figure 1). Pop 2 presenting Dayyer population had lower area (Table 5; Figure 1).

### DISCUSSION

The aim of this study was to evaluate how much genetic diversity exists throughout different habitats of *A. marina* in Bushehr Province (located in south of Iran). Overall, the patterns support the notion that the species is not completely outcrossing (correspond to F<sub>IS</sub>=0.1605 for overall loci), while according to the findings of Maguire studies, those populations at extremes of the worldwide

**Table 5.** Genetic identity (upper diagonal) and Genetic distance (lower diagonal) Nei.

Population ID	pop1 (Bordekhoon)	pop2 (Dayyer)	pop3 (Asaluyeh)
pop1 (Bordekhoon)	****	0.787	0.8876
pop2 (Dayyer)	0.2396	****	0.8480
pop3 (Asaluyeh)	0.1192	0.1649	****

**Figure 1.** Population clustering of Bordekhoon, Asaluyeh and Dayyer using the program popgene 32, Nei coeff and UPGMA program.

range confirmed the species have high levels of inbreeding (Maguire et al., 2000b), and corresponds to previous data using allozymes (Duke et al., 1998). The average level of heterozygosity (0.589) for Bushehr habitats is not reduced. This is not unexpected as these populations have not been subjected to repeated bottleneck or founder effects in earlier time, due to episodes of glaciations and transgression (Saenger 1998). On the other hand, mangrove habitats of Iran are located on global belt of mangrove forests. Therefore in comparison to some populations (like Japan, Victoria, Western Australia, New South Wales and South Africa) that showed reduced levels of heterozygosity, Bushehr habitats individuals have appropriate diversity. This issue strengthens the idea of conserving the genetic variation of remaining populations. The results suggest finding evolutionary geographic center of mangrove forests of Iran existing in the other provinces with more longitude, then rehabilitating these populations by those original individuals. The habitats were exposed to oil pollutions in Iraq-Kuwait war, which might have led to changes in the genetic variations of *A. marina*. In fact, lots of pollutants remained on resistant genotypes of the species and destroyed a lot of particular mangrove fauna leading to more inbreeding. Overall results indicate that genetic differences among the three shorelines were greatly influenced by geographic distances, isolation, and thus limitations in gene flow. Dayyer mangroves had lower amount of genetic variation (Figure 1). Asalooeyeh is attached to the best mangrove habitats on the Persian Gulf and Oman Sea, so it is not unexpected that this population may have been subjected to more gene exchange with neighbors.

In this manner, decreasing effective population size will

lead to high inbreeding and genetic purity of the habitat, and then will increase ecosystem susceptibility versus biological impressions. Three years after completion of this study, physiological weaknesses appeared and sudden drying of almost a half percent of tress in the smallest habitat with no specific disease symptoms occurred. A survey can leading to the natural ecosystems to be evaluated is required to adopt necessary managing plans against the human-made phenomena.

Since the mangrove forests of the southern regions of Iran are located on the global belt of mangrove forests, and may have not been subjected to the environmental impacts (Saenger, 1998), and with regards to its average level of genetic variation as well, it could be inferred that the forests are settled in an appropriate state compared to *A. marina* in the entire worldwide range. But appearance of new oil pollution centers (like huge oil refineries) close to the most various habitats (Asalooeyeh) accompanied by heavily exploitation of foliage for domesticated animals (especially in Dayyer) may cause some pressures leading to the loss of genetic diversity in the mangrove ecosystems.

With due attention to a previous study (Maguire et al., 2000b), United Arab Emirates (Abu Dhabi) mangrove forests (located very close to Iran) have eight private alleles. It will not be surprising if the mangrove forests of the Persian Gulf have alleles unique to Iran, as well. Therefore, protection of the *A. marina* habitats in the frame of reserved area in Man and Biosphere program (M and B) would be of primary priority in Iran because environment constrains, as well as ecological factors may also have a significant effect on the levels of heterozygosity, inbreeding and population structure.

Microsatellite variation has been found to be generally

high in many plant species. Although the number of alleles detected in a study is dependent on the sample size, it gives an indication of the degree of variability detected with microsatellite loci. In this study, three microsatellite loci were examined and found to be highly polymorphic with gene diversity estimates based on only three populations. Large number of individuals will provide more accurate estimates of gene diversity. The current estimate is sufficiently high to indicate that microsatellites will be very useful for genetic analysis of *A. marina* and related species. Microsatellite are an efficient means of individual identification and can provide high resolution and sensitivity for the estimation of genetic parameters essential for assessing the effects of deforestation and forest fragmentation in mangrove communities. The need to understand what determines the patterns of genetic variation is an important issue for mangrove conservation and reforestation/aforestation schemes. Most species which occupy environmentally sensitive habitats in both temperate and tropical regions, such as mangroves, are out-breeders. For these species, information on the extent of gene flow, genetic differentiation, and levels of inbreeding (particularly in fragmented population) has important practical relevance for the conservation of genetic resources. The ease of using microsatellite markers make their use preferable, particularly when analyzing large numbers of individuals with small numbers of loci. Due to their inherent attributes, microsatellites can be readily employed for these purposes. This will support improved conservation and sustainable management of our ever declining mangrove genetic resources.

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