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First Microsatellite Loci of Red Mullet (*Mullus barbatus*) and Their Application to Genetic Structure Analysis of Adriatic Shared Stock

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Abstract: In order to study the genetic structure of the Adriatic shared stock of red mullet (*Mullus barbatus*), we developed a set of dinucleotide microsatellite markers. A dinucleotide-enriched genomic library was obtained, and 6 polymorphic dinucleotide loci were successfully optimized. The markers showed high expected heterozygosity (from 0.68 to 0.92) and allele number (from 12 to 33); thus they appear to be suitable for detecting genetic differences in the population of red mullet. Four Adriatic samples were subsequently analyzed for microsatellite variation, and the results showed subtle but statistically significant genetic differentiation, indicating that the Adriatic red mullet may group into local, genetically isolated populations. No correlation between geographic distance and genetic differentiation was observed. In addition, the evidence of recent bottlenecks in the Adriatic samples indicates that the observed population subdivision might reflect random local allelic variations, generated by reproductive success, survival rates, or fishing pressure.

Key words: *Mullus barbatus*, microsatellites, Adratic Sea, genetic structure.

INTRODUCTION

Microsatellites are nuclear codominant DNA markers consisting of short tandem repeats of 1 to 6 bp that appear to be highly abundant and dispersed throughout the genome of eukaryotes (Tautz, 1989). Variation in the number of repeat units often produces an abundance of alleles distinguishable by molecular size. Because of their high level of polymorphism, microsatellite markers have been useful in genetic studies of population structure, particularly in organisms showing low levels of differentiation such as marine fishes (Estoup et al., 1998). These molecular tools have been used for indirect monitoring of demographic features of shared marine fishery stocks, achieving useful scientific data for the improvement of fisheries and resource conservation (for example, see Ruzzante et al., 1998; Nesbø et al., 2000; Hauser et al., 2002).

Within the framework of a regional program funded by FAO and the Italian Ministry of Agriculture and Forestry Policies (AdriaMed 2000), two genetic research projects were recently financed to develop polymorphic, species-specific microsatellites and investigate the popula-

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tion structure of 15 species considered of high priority for fisheries in the Adriatic Sea. Among them, the red mullet (*Mullus barbatus*, Mullidae) is one of the most economically important fisheries resources shared by the Adriatic countries. This benthic species is widely distributed around the Mediterranean basin and is abundant on muddy grounds in a depth range of 5 to 100 m (Relini et al., 1999).

Using an enrichment protocol, we developed a suite of 6 polymorphic microsatellite loci specific for the red mullet and used them to detect subtle but significant genetic variability among red mullet populations collected from the Adriatic Sea.

Materials and Methods

Samples of *Mullus barbatus* were collected in 4 locations of the Adriatic Sea (Figure 1) during the autumn-winter MEDITS 2001 scientific trawl survey. Individual genomic DNA was extracted from 25 to 50 mg of dried tissue according to a saline procedure (Miller et al., 1988).

A $(GT)_n$-enriched library was obtained using the FIASCO protocol (Zane et al., 2002). After extraction, genomic DNA was simultaneously digested with *Mse*I and ligated to *Mse*I-adapter (5′-TACTCAGGACTCAT-3′/5′-GAC GATGAGTCCTGAG-3′). Restricted and ligated fragments were amplified with *Mse*I adapter-specific primers, hybridized with a biotinylated $(AC)_{17}$ probe, selectively captured by streptavidin-coated beads (Roche), and separated by a magnetic field. DNA was eluted from the bead probes, reamplifled with *Mse*I adapter-specific primers, and cloned using the TOPO-TA cloning kit (Invitrogen) following the manufacturer's protocol. The recombinant clones were sequenced using the ABIPrism BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and resolved on an ABI310 Genetic Analyser (Applied Biosystems). Polymerase chain reaction (PCR) primers were designed using Primer3 software (Rozen and Skaletsky, 1998) and optimized for PCR amplification testing over a range of annealing temperatures and $MgCl_2$ concentrations.

The PCR reactions were performed on a GeneAMP PCR System 9700 (Applied Biosystems) in 10 µl total volume, which included about 20 ng of genomic DNA, 1.5 to 2.5 mM $MgCl_2$ (Table 1), 0.5 µM of each primer, 200 µM dNTPs, 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, and 1 U of *Taq* polymerase (Promega). Individuals were genotyped by assessing the allele size on an ABI310 automated Genetic Analyser (Applied Biosystems), using forward primers labeled with 6-FAM, HEX, or TAMRA (MWG Biotech) and ROX 400 (Applied Biosystems) as internal standard. Allele sizing was performed using GeneScan Analysis Software Version 2.02 (Applied Biosystems).

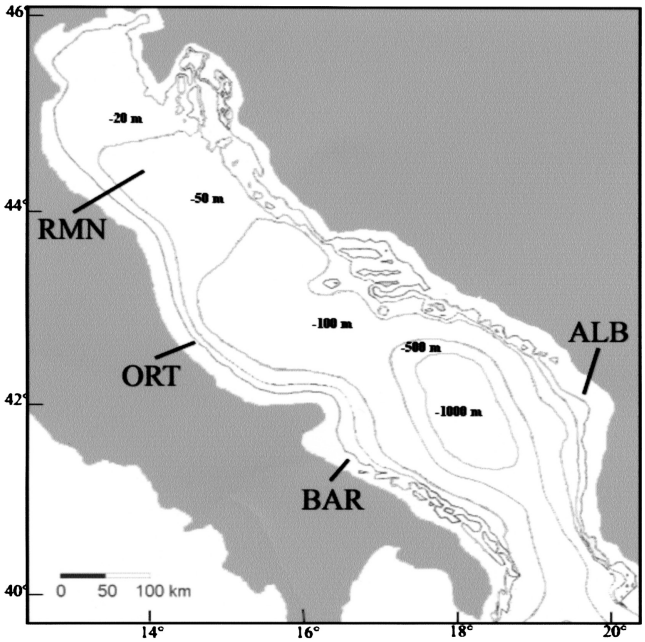

Figure 1. Map of the red mullet samples sites in the Adriatic Sea. ALB (Albania), BAR (Bari), ORT (Ortona), RMN (Rimini).

Microsatellite polymorphism within samples was measured as the mean number of alleles per locus, and observed and unbiased expected heterozygosity were determined using the Genetix 4.0 software package (Belkhir et al., 1996). The tests for linkage disequilibrium between pairs of loci was performed using Genepop 3.1 (Raymond and Rousset, 1995). Deviations from Hardy-Weinberg equilibrium (HWE) were tested using Fisher's exact test, with the level of significance determined by a Markov chain method using Genepop3.1 software (Raymond and Rousset, 1995). Sequential Bonferroni corrections (Rice, 1989) for multiple comparisons were applied when necessary. The level of genetic differentiation among Adriatic samples was investigated using two different statistical tests. The Spagedi software (Hardy and Vekemans, 2002) was used to calculate the overall and the pairwise Wright's F_{ST} (Weir and Cockerham, 1984), and the Fisher's exact test implemented in Genepop 3.1 software (Raymond and Rousset, 1995) was applied to test samples for simple frequency differentiation. The results were tested for significant departure from zero

Table 1. Amplication Conditions of Six Microsatellite Loci in the Red Mullet

Locus	Primers 5′-3′	Repeat	T_a(°C)	[$MgCl_2$] (mM)	GenBank Acc. No.
Mb6	F:TCTCCCATCATAAACAG CAAAR:GGGAAATAGCTC AGGTTCGT	$(CA)_3AA(CA)_{10}$	52	1.5	AY241266
Mb7	F:CATTTGTCCCCCTCAGTG TAR:TTTCTGCTCGGTTAGTGGTC	$(AC)_3AG(AC)_9$	52	1.5	AY239606
Mb15	F:AGGAGCAGGAGGTCTT CATCR:TCCTGAGTAAGACACGACCAG	$(CA)_5TG(CA)_3$ $TG(CA)_5TG(CA)_8$	52	2.5	AY239608
Mb26b	F:CCTTCCTTTATCGTTCTCT CTGGR:CCACATTTTATCGCAACTTTCA	$(CA)_{16}$	52	2.5	AY241875
Mb39	F:TGCAGTATTTCTCGGATTG CR:TCAAGAGACGTGCCAGAATG	$(GT)_8(CGGT)_{10}$	52	2	AY351668
Mb31	F:AGCGCCGAGTATAACAGAC AATR:CTTACTCAACGAGTGTTTGATCG	$(CA)_{22}$	52	1.5	AY239609

using the permutation tests available in the respective software packages. The likehood of individual's membership in each sample was calculated from their multilocus genotypes using the jackknife procedure implemented in WHICHRUN 4.1 software (Banks and Eichert, 2000).

We tested whether the red mullet samples had experienced a recent reduction of their effective population size using the Wilcoxon test implemented in BOTTLENECK software (Cornuet and Luikart, 1996), to assess the probability of fit to random-drift equilibrium for loci evolving under the infinite allele module (IAM). To test for isolation by distance between samples, the Mantel tests was performed using GENEPOP 3.1 software (Raymond and Rousset, 1995).

RESULTS

More than 200 clones containing potential dinucleotide microsatellite loci were obtained. Of 40 sequenced clones, 27 (67.5%) contained microsatellite sequences. Among them 10 were perfect, 9 imperfect, and 8 compound. All the microsatellites contained GT/AC repeats, 26 were dinucleotide, and only 1 was composed of GT and GCGT repeats. For 10 loci that contained suitable flanking sequences, PCR primer pairs were designed and tested on 10 individuals over a range of annealing temperatures and $MgCl_2$ concentrations. Six loci reliably amplified a specific polymorphic product (Table 1), while the remaining 4 loci showed either nonspecific products or lack of amplification.

Allele variation at the 6 microsatellite loci was further examined in 206 red mullets belonging to 4 geographical samples of the Adriatic Sea (Figure 1). Genetic variability, reflected in the degree of heterozygosity and number of alleles, was high (Table 2). Unbiased mean expected heterozygosity ranged from 0.68 from 0.92 (mean, 0.83), observed heterozygosity ranged from 0.67 from 0.88 (mean, 0.78), and the number of alleles scored at each locus ranged from 12 to 33 (mean, 18.3). The test for linkage disequilibrium between pairs of loci showed no significant *P*-values. The observed genotype frequencies of the 4 samples were tested with the respect to HWE (Table 2). There was a slight overall tendency for deficiencies of heterozygotes: 5 of 24 single-locus tests showed significant departure from HWE; however, after sequential Bonferroni's correction only 3 remained significant, and they were never associated with a particular locus or sample.

The genetic differentiation among Adriatic samples was estimated using *F* statistics and the exact test for homogeneity of allelic frequencies. Both estimates indicated spatial genetic heterogeneity among the Adriatic stock. The overall F_{ST} value was low but significant (0.005, $P < 0.01$), and the Fisher's exact test for homogeneity of allelic frequencies indicated significant genetic differences among samples ($P < 0.001$). Similarly, two F_{ST} pairwise comparisons and four Fisher's exact tests of population differentiation showed significant differences in the allelic structure of samples (Table 3). The single-locus F_{ST} estimates ranged from 0.001

Table 2. Genetic Variation at Six Microsatellite Loci in *Mullus barbatus* Samples from the Adriatic Sea[a]

	Samples (n)[b]				
Locus	RMN (58)	ORT (47)	BAR (48)	ALB (53)	Mean[c]
Mb6					
Allele size	202–226	200–216	202–236	202–226	
N_a	8	8	9	11	9.0 (13)
H_e	0.63	0.70	0.69	0.69	0.68
H_o	0.66	0.62	0.71	0.72	0.67
Mb7					
Allele size	166–182	152–182	166–180	164–184	
N_a	9	10	8	11	9.5 (12)
H_e	0.83	0.87	0.86	0.87	0.86
H_o	0.81	0.79	0.83	0.83	0.82
Mb15					
Allele size	249–287	263–295	249–307	269–291	
N_a	9	12	12	10	10.8 (19)
H_e	0.76	0.80	0.81	0.80	0.79
H_o	0.74	**0.66**	0.60*	0.85	0.71
Mb26b					
Allele size	227–249	229–249	231–255	229–247	
N_a	11	11	10	10	10.5 (14)
H_e	0.85	0.87	0.84	0.81	0.84
H_o	0.84	0.81	0.65**	0.82	0.78
Mb31					
Allele size	104–138	116–160	114–140	116–444	
N_a	14	16	13	14	14.2 (19)
H_e	0.80	0.90	0.87	0.86	0.86
H_o	0.78	0.96	0.94	0.85	0.88
Mb39					
Allele size	271–335	289–341	271–345	289–229	
N_a	26	23	22	19	22.5 (33)
H_e	0.93	0.92	0.91	0.90	0.92
H_o	0.93	0.70***	0.81	**0.77**	0.80

[a]Number of individuals (n), number of alleles (N_a), unbiased expected heterozygosity (H_e), and observed heterozygosity (H_o) are given.
[b]Significant departure from the HWE after sequential Bonferroni's correction is denoted as follows: *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$. Values in bold were not significant after sequential Bonferroni's correction.
[c]Values in parentheses indicates the total number of alleles for each locus across all populations.

(*Mb6*) to 0.012 (*Mb31*). All the loci contributed to the genetic heterogeneity of samples (Table 3), even if none appeared in all the significantly different comparisons.

To further investigate whether the samples represented genetically distinct populations, we calculated the proportion of individuals assigned to the site of capture with the proportion assigned to different sites, on the basis of individual multilocus genotypes. The results showed that the fraction of individuals assigned to the site of sampling—respectively, 49% for RMN, 57% for ORT, 41% for BAR, and 40% for ALB—was much higher than the fractions assigned to other sites. Hedrick (1999) suggested that low levels of genetic differentiation at microsatellite loci may be statistically significant but not represent biologically meaningful differences if the analyzed samples showed indications of recent reduction in population size. A significant indication of population bottleneck was found for the RMN, ORT, and BAR samples ($P < 0.05$), suggesting that this may have affected the measure of genetic differentiation.

Table 3. Pairwise Estimates of F_{ST} (below diagonal) and Allelic Differentiation (above diagonal) Between Red Mullet Adriatic Samples[a]

	RMN	ORT	BAR	ALB
RMN	—	***(*6**, *7**, *26b**, *31**, *39**)	NS	*(*7**, *15**)
ORT	0.011**	—	**(*6**, *39**)	***(*6**, *39**)
BAR	0.002	0.002	—	—
ALB	**0.007**	0.008*	0.001	—

[a]Significance after sequential Bonferroni's correction is denoted as follows: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant. Values in bold were not significant after sequential Bonferroni's correction. The loci that showed significant allelic differentiation for each comparison are given in parentheses.

Despite the evidence of genetic differentiation among Adriatic samples, there was no indication of isolation by distance; in fact, the result of the Mantel test of isolation by distance was not significant ($P = 0.256$).

Discussion

In this study we used the FIASCO protocol (Zane et al., 2002) to obtain an enriched dinucleotide microsatellite library from the red mullet (*Mullus barbatus*). The frequency of positive clones found in the red mullet microsatellite library was high (67.5%), quite similar to that obtained in the black-bellied angler (74%) using the same protocol (Garoia et al., 2003). Mean heterozygosity and allele number of microsatellite loci developed for the red mullet are consistent with the corresponding values detected in other teleost fish (De Woody and Avise, 2000). Because of the high levels of polymorphism detected, these loci appear to be useful markers for analyzing genetic stock structure of red mullet populations in the Adriatic.

The red mullet microsatellite loci showed high and homogeneous genetic variability across the Adriatic samples, with a slight overall tendency for deficiency of heterozygous genotypes. Overall, the samples did not show significant departures from HWE, even though 3 single-locus tests revealed significant heterozygote deficiency. Relevant heterozygote deficiencies have been reported for many fish species (Waldman and McKinnon, 1993; Hoarau et al., 2002) and could result from null alleles, population substructuring, or selection. The lack of null amplifications in the 206 individuals we analyzed suggests that the presence of null alleles was an unlikely cause of the deficiency in this data set. The Wahlund effect is the explanation most frequently invoked for observed heterozygote deficiencies. Individual red mullet, after a pelagic larval phase, move toward sandy coastal areas and adopt a benthic life style (Relini et al., 1999). This migration could cause the admixture of individuals from genetically different populations (see below) and contribute to the observed departures from HWE.

Both the estimators used to test the extent of genetic differentiation within the red mullet Adriatic stock revealed significant overall heterogeneity. The level of differentiation showed by the exact test of allelic differentiation was higher than that assessed by F_{ST} ($P < 0.001$ and $P < 0.01$, respectively). This discrepancy may be due to the fact that Fisher's exact test gives more weight to rare alleles and thus is more sensitive for detecting subtle genetic differences among populations. The number of significant pairwise comparisons was higher using the exact test than using the *F*-statistics, but the RMN-ORT and ORT-ALB comparisons showed higher differentiation using both estimators. Furthermore, the higher fraction of individuals assigned to the site of sampling rather than to other sites, on the basis of the multilocus genotypes, supports the observation that the samples represent genetically distinct stocks. These results indicate that the red mullets may group into local subpopulations that could remain partly isolated from each other.

There are several possible explanations for a similar pattern of differentiation independent of distance. Such a pattern could be explained by the passive dispersion of larvae with the marine currents, rather than active migration of adult individuals (Knutsen at al., 2003), or with recent colonization events (Slatkin, 1993). Moreover, the evidence of recent population size reduction in the red mullet Adriatic samples suggests that the genetic differentiation could also be generated from random changes in allele frequencies (Hedrick, 1999). Previous studies showed large seasonal fluctuation in red mullet catches, and a strong increase in the catches was observed in fall surveys after the summer trawl ban, indicating that fishing pressure could contribute to the stock size fluctuation (Manfrin et al., 1998; Marano et al., 1998). Thus the preliminary data on microsatellite variation suggest the occurrence of genetic

structure in the Adriatic shared stock of the red mullet. However, further analyses of fine-scaled samples from Adriatic feeding and spawning grounds, as well as from temporal replicates, will be needed to confirm the stability of genetic structuring.

Failure to identify distinct populations could lead to overexploitation and decline of the marine resources (Hutchings, 2000; Hauser et al. 2002), so caution should be taken regarding fisheries and conservation of the Adriatic red mullet.

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