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ASSESSMENT OF INBREEDING IN HATCHERY SAMPLES OF THE RED SEABREAM *PAGRUS MAJOR* (*Perciformes Sparidae*)

RIASSUNTO

Valutazione dell'inbreeding in campioni d'acquacoltura di Pagrus major. Negli impianti di acquacoltura, il monitoraggio genetico degli stock allevati è necessario per controllare che i ripetuti incroci tra gli individui non diminuiscano la variabilità genica delle popolazioni favorendo l'azione di geni recessivi dannosi. In questo lavoro, è stato determinato il livello di variabilità genica della specie *Pagrus major* (famiglia Sparidae) in una popolazione selvatica (*wild-type*) proveniente dalle coste del Giappone (Nagasaki) e in due campioni di impianti di acquacoltura (Hyogo, Giappone, e Petrosino Trapani, Italia). Sono stati utilizzati tre loci di microsatelliti: *Pma3, Pma4, Pma5*. Il locus *Pma4* ha mostrato il grado di variabilità più basso, in termini di eterozigosità osservata, pur non avendo il più basso numero di alleli, rilevato, invece, nel locus *Pma5*. L'eterozigosità osservata variava da un valore minimo di 0.27 (nel campione di pesci allevati giapponesi) ad un valore massimo di 0.86 (nel campione di allevati italiano) e l'eterozigosità attesa da 0.087 a 0.95. Nel complesso, tutti gli *stock* di pesci allevati e selvatici hanno mostrato un *deficit* significativo di eterozigoti. Il *deficit* di eterozigoti, normalmente riscontrato nei loci di microsatelliti, può essere dovuto alla presenza di alleli nulli, determinati da mutazioni nel sito di *priming*. Nel nostro caso la deviazione dall'equilibrio di Hardy-Weinberg è stata spiegata da un effetto di *inbreeding* nei campioni allevati. Lo stesso effetto si può supporre nel campione selvatico poiché nell'area giapponese i giovanili allevati vengono costantemente rilasciati in mare, creando un mescolamento tra stock naturale e allevato. Queste ipotesi vengono supportate dal calcolo dell'AMOVA, che ha mostrato una ripartizione della varianza molecolare del 96% all'interno dei campioni e del 4% nel confronto tra le popolazioni. Il test di differenziazione genetica tra le popolazioni, elaborato con l'indice F_{ST}, il cui valore medio era 0.037, ha dato valori significativi in ogni confronto a coppie fatto tra i campioni. I valori di distanza genetica, hanno mostrato che i campioni giapponesi si raggruppavano insieme, mentre il campione siciliano di Petrosino risultava essere più distante, in accordo con la distanza geografica dei campioni.

SUMMARY

Red sea bream *Pagrus major*, belonging to the Sparidae family, commonly called "madai" in Japanese, is distributed along the coastal waters of Japan where it is one of the most valuable and popular fishery resources. *Pagrus major* is, furthermore, an important species in asiatic hatcheries and its production is expanding in European farms too. In the present study two hatchery populations, an Italian one and a Japanese population from Hyogo, were compared to a wild population originating from Japan (Nagasaki) to estimate their level of genetic polymorphism. We chose analysis of variation of microsatellites loci because they are traditionally considered an informative marker in revealing the genetic divergence between populations. Three microsatellites loci were tested. *Pma3, Pma4* and *Pma5*. AMOVA analysis revealed a high portion of variance within each population and a low portion of variability between samples confirming that the three samples originated from the same gene pool. In particular, the genetic distance grouped together the two Japanese samples which both diverged with the highest values from the Italian sample. The deficit of heterozygosity could not have been attributable only to the presence of null allele, but to inbreeding, as demonstrated by the observation of homozygotes frequency much higher than the expected null homozygotes frequency, calculated by two different indexes to estimate of null allele frequency.

INTRODUCTION

Red sea bream *Pagrus major*, belonging to the Sparidae family, commonly called "madai" in Japanese, is distributed along the coastal waters of Japan where it is one of the most valuable and popular fishery resources. *Pagrus major* is, furthermore, an important species in asiatic hatcheries and its production is expanding in European farms too.

Population structures of various fish species have been studied by multilocus DNA fingerprinting, particularly by isolation of microsatellite loci. We chose analysis of variation of microsatellites loci because they are traditionally considered an informative marker in revealing the genetic divergence between populations. Microsatellites consist of tandem arrays of di, tri, or tetra nucleotide sequences, flanked by regions of unique DNA sequences, and have been shown to be highly polymorphic in fish (O' CONNEL & WRIGHT, 1997).

The high variability and accuracy of assaying microsatellites make them the marker of choice for resolution population analysis (WRIGHT & BENZEN, 1995). Genetic tagging using microsatellite markers is an adequate method to recognize released fish present in the wild and to pedigree individuals in the same area over a longer period of time.

The understanding of the processes and organization of genetic diversity of various fish species is essential for the sustainable management of exploited species. It is noteworthy that a loss of genetic variability within stock can occur in hatchery populations as a result of inbreeding, selective breeding or domestication (ALLENDORF & PHELPS, 1980). Regular monitor-

ing of levels of variation in hatchery stocks is necessary to check the genetic polymorphism. Such monitoring compares a hatchery sample with a second sample, collected either in a previous generation or from the parental wild stock (WARD & GREWE, 1995). In particular, in Japan, because of a steady decrease in production of *Pagrus major*, a stock enhancement program was started in several locations with the release of juveniles reared in governmental hatcheries. Several authors have discussed the potential risks associated with stock enhancement practices (BLANKENSHIP & LEBER, 1995), in fact the genetic constitution of hatchery populations is totally different from that of wild populations (usually reduced genetic variation).

In the present study, two hatchery populations, an Italian one and a Japanese population from Hyogo, were compared to a wild population originating from Japan (Nagasaki) to estimate their level of genetic polymorphism.

The Italian hatchery population, which comes from Petrosino (Sicily, Southern Italy), originates from Japanese eggs that were bought fifteen years ago; we also conducted the microsatellite analysis addressed to the characterization of an Italian hatchery population which has never been studied from the genetic point of view.

MATERIALS AND METHODS

Samples of the red bream *Pagrus maior*, were collected from three locations: Nagasaki (Japan), where a wild sample was caught in 1996; Hyogo Prefecture Fisheries Experimental Station (Japan), where hatchery samples were caught in 1999; Petrosino (Sicily, Southern Italy), where hatchery samples were caught in 2001 (Fig. 1). The total number of analyzed individuals is 170. DNA was extracted from ethanol-preserved skeletal muscle tissue by standard SDS-phenol/chloroform procedures and stored at 4 °C, prior to PCR analysis.

PCR analysis was carried out using a Thermal cycler PTC 100 (*Genenco*). The 20 *µl* of reaction contained: 15/30 *ng* of template DNA; 2.5 *µM* each unlabelled primer; 0.25 *µM* fluorescent labelled forward primer; 1.2 *mM* $MgCl_2$; 20 *mM* each dNTPs and 0.25 unit of *Taq* Gold Polymerase (*Perkin Elmer*). Specific primers were used to amplify three microsatellite loci (*Pma3, Pma4* and *Pma5*; TAKAGI *et al.*, 1997): *Pma3*, Forward 5'-GGTTTAGCAAGAGAAAGGG-3', Reverse 5'- TAAACATGACAAACTGAGGTG-3'; *Pma4*, Forward 5'-GTTGGCTCGGTCTAAAGTC-3', Reverse 5'-TCTCCACTCCGTATTGCTC-3'; *Pma5*, Forward 5'-TCGGATTGAGTATCTGTGGG-3', Reverse 5'-AGGTTCTCCGTCACTGTCC-3'. Samples were

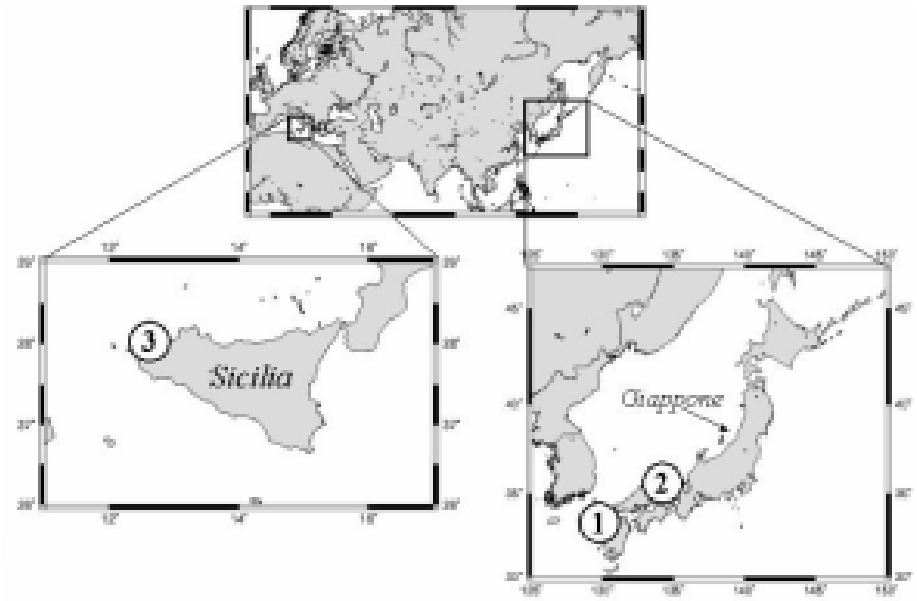

Fig. 1 — Sample sites: 1) Hyogo, Japan; 2) Nagasaki, Japan; 3) Petrosino, Italy.

amplified using the following cycle parameters: an initial denaturation step at 98 °C for 10 *min*; 7 cycles 1 *min* at 94 °C, 30 *sec* at the appropriate annealing temperature, 30 *sec* at 72 °C; 33 cycles 30 *sec* at 90 °C, 30 sec at annealing temperature, 30 *sec* at 72 °C. The size of the fluorescent-labeled DNA fragments were determined in a automated sequencer ABI PRISM 3700 (*Applied Biosystems*).

Comparative measures of genetic diversity for each sample were calculated in the form of allelic diversity: total number of alleles, mean number of alleles per locus and private alleles. Hardy-Weinberg equilibriun was evaluated for all loci across all populations and to estimate gene flow among samples F_{ST} values were calculated.

Allelic frequencies were computed by using FSTAT (version 2.9.3.1; GOUDET, 1995). Genetic diversity for each sample was measured as the number of alleles per locus, the observed (*Ho*) and expected (*He*) heterozygosity, using ARLEQUIN 2.000 (EXCOFFIER, 2000) and FSTAT (version 2.9.3.1; GOUDET, 1995). To assess conformity of genotype distributions with Hardy-Weinberg expectations a probability test, for each locus and on separate sample, was performed according to the Markov chain procedure, using GENEPOP software version 3.1 (RAYMOND & ROUSSET, 1995).

Two methods were applied for estimating null allele frequency from het-

erozygote deficiency: the first, named here r_C, was proposed by CHAKRABORTY *et al.* (1992), the second one, r_B, by BROOKFIELD (1996).

To determine population genetic structure at the three locations, ARLEQUIN software was used to test the significance of pairwise Slatkin's linearized F_{ST} (SLATKIN, 1995) using random allelic permutation procedure and to perform hierarchical AMOVA (Analysis of Molecular Variance) to partition of genetic diversity within and between populations (EXCOFFIER *et al.*, 1992; EXCOFFIER, 2000).

To examine the distribution of heterogeneity among the samples of both species, genetic distances (NEI, 1978) were calculated and clustered by Neighbor-Joining algorithm using the software GDA (Genetic Data Analysis; LEWIS & ZAYKIN, 2001).

RESULTS AND DISCUSSION

Three microsatellites loci were tested and scored polymorphic at the level of frequency 0.95 for the most common allele. We were able to reveal 19 alleles for the *Pma3* locus, 18 for the *Pma4* locus and 16 for the *Pma5* locus. Allelic frequencies were computed and are shown in Table 1. Allele size ranged from 81 bp to 121 bp at the locus *Pma3*, from 97 to 169 bp at locus *Pma4* and from 113 to 151 bp at the locus *Pma5*. Allelic length was in accordance with the range of allelic variants previously found by PEREZ-ENRIQUEZ & TANIGUCHI (1999).

AMOVA analysis revealed a high portion of variance within each population and a low portion of variability between samples (Table 2) confirming that the three samples originated from the same gene pool. In particular, the genetic distance (NEI, 1978) grouped together the two Japanese samples (Table 3) which both diverged with the highest values from the Italian sample.

Moreover, there were significant differences in allelic frequency distribution among the samples as showed by the pairwise differentiation test that showed the samples distinguishable from each other, since all the F_{ST} values appeared to be significantly different from zero (Table 3).

Observed heterozygosity for all loci among all samples varied from 0.27 to 0.86, while expected heterozygosity ranged from 0.84 to 0.94. *Pma4* locus showed the lowest level of observed heterozigosity especially in the Japanese hatchery sample (0.27). A highly significant deficit of heterozygosity was observed for *Pma3* and *Pma4* locus in all the examined populations; the significance of this departure from Hardy-Weinberg equilibrium was maintained after Bonferroni procedure, which diminished the level of p from 0.05 to 0.005.

Table 1
Allele frequencies, observed (Ho) *and expected* (He) *heterozygosity of red sea bream at the three locations* (N = *sample size)*

	Pma3 Wildtype	Hatchery			Pma4 Wildtype	Hatchery			Pma5 Wildtype	Hatchery	
	Japan	*Japan*	*Italy*		*Japan*	*Japan*	*Italy*		*Japan*	*Japan*	*Italy*
N	60	50	60	N	60	50	60	N	30	25	30
Allele				Allele				Allele			
121	*0.019*	*0*	*0*	169	*0*	*0.095*	*0*	151	*0*	*0.020*	*0.155*
119	*0.037*	*0.024*	*0.019*	149	*0*	*0.095*	*0*	149	*0.086*	*0.040*	*0.017*
117	*0*	*0.048*	*0*	133	*0*	*0.048*	*0*	147	*0.138*	*0.120*	*0.069*
115	*0*	*0.024*	*0.019*								
113	*0.056*	*0.071*	*0.093*	131	*0.077*	*0.095*	*0*	145	*0.069*	*0.100*	*0*
111	*0.056*	*0*	*0.019*	123	*0.019*	*0*	*0*	143	*0.034*	*0.060*	*0.017*
109	*0*	*0*	*0.019*	121	*0*	*0.024*	*0*	141	*0.017*	*0.020*	*0.138*
107	*0.056*	*0*	*0.074*	119	*0.019*	*0*	*0*	139	*0*	*0*	*0.121*
105	*0.093*	*0.190*	*0.056*	117	*0*	*0*	*0.036*	137	*0*	*0.060*	*0*
101	*0.056*	*0*	*0.037*	115	*0.038*	*0.024*	*0*	135	*0.034*	*0.020*	*0.034*
99	*0.056*	*0.024*	*0.019*	113	*0.058*	*0*	*0.125*	133	*0.241*	*0*	*0.103*
97	*0.037*	*0.071*	*0.167*	111	*0.115*	*0.071*	*0.268*	131	*0.103*	*0.080*	*0.103*
95	*0.074*	*0.048*	*0*	109	*0.154*	*0*	*0.018*	129	*0.172*	*0.180*	*0.155*
93	*0*	*0.190*	*0.019*	107	*0.192*	*0.167*	*0.179*	127	*0.052*	*0.140*	0
91	*0.111*	*0*	*0.222*	105	*0.077*	*0*	*0.018*	125	*0.052*	*0*	*0*
89	*0.056*	*0*	*0*	103	*0.077*	*0.095*	*0.196*	123	*0*	*0.120*	*0.086*
85	*0.111*	*0*	*0.204*	101	*0.058*	*0.190*	*0.036*	113	*0*	*0.040*	*0*
83	*0.148*	*0.167*	*0.037*	99	*0.058*	*0*	*0.05*				
81	*0.037*	*0.143*	*0*	97	*0.058*	*0.095*	*0.071*				
Ho	*0.75****	*0.62****	*0.65****		*0.42****	*0.27****	*0.68****		*0.70***	*0.75**	*0.86*
He	*0.94*	*0.92*	*0.87*		*0.92*	*0.92*	*0.90*		*0.88*	*0.84*	*0.90*

(Departure from Hardy-Weinberg equilibrium; Markov chain procedure, * $p < 0.05$, ** $p < 0.005$, *** $p = 0.000$*)*

Table 2
Hierarchical analysis of molecular variance for Pagrus major using microsatellite markers.

Sorce of variation	Degree of freedom	Sum of square	Variance components	Percentage of variation
Among populations	2	2.656	0.01600 *Va*	3.63
Within populations	167	70.850	0.42425 *Vb*	96.37
Total	169	73.506	0.444025	

Fixation Index F_{ST}: 0.03635

Table 3
Genetic data analysis: values of F_{ST} (above the diagonal) and standard genetic distance (Nei, 1978) (below the diagonal).

Pagrus major samples	*Wild-type* Japan	*Hatchery* Japan	Italy
	Wild-type Japan	–	0.036*
0.040****Hatchery* Japan	0.268	–	0.031***

(Permutation test, * $p < 0.05$, *** $p = 0.000$)

Usually the deficit of heterozygotes in microsatellites loci may be explained by the presence of presuntive null alleles, or inbreeding units and a reduction in population size as a result of the hatchery activity. The presence of null alleles determines a phenomenon such that many of the apparent homozygotes are, in reality, heterozygotes between a visible and a null allele (PEMBERTON *et al.*, 1995). Such genotypes will appear to be homozygotes since they show a single band on the electropherogram. For microsatellites, such null alleles can arise when mutations prevent primers from binding (CALLEN *et al.*, 1993). Here we report a calculation of estimate of null allele frequency from two indexes proposed by CHAKRABORTY *et al.* (1992) and BROOKFIELD (1996) and here named, respectively, $r_C = (He - Ho) / (He + Ho)$ and $r_B = (He - Ho) / (1+He)$. For each sample, the value of r was used to estimate the expected null homozygotes frequency, that is the individuals where no PCR band was scored.

Frequencies of estimates of null heterozygotes according to r_C and r_B formulas and frequencies of observed null homozygotes resulting in no-bands at the three locations are listed in Table 4. In the most cases the number of expected null homozygotes was much lower than the observed, indicating that the deficit of heterozygosity could not be attributable only to the presence of null allele, but to inbreeding.

The same effect should be supposed regarding the wild type sample, since it should be noted that Japanese hatchery-reared fish are usually released in the natural populations, creating a mixture between hatchery and wild gene pools (PEREZ-ENRIQUEZ & TANIGUCHI, 1999).

Loci of inbreeding populations tends to saturate, that is to create populations with identical specimens. In our case, since the locus *Pma5* showed a lower departure in heterozygosity deficit, this locus seems not to be so close to saturation, as *Pma3* and *Pma4* do, and can be considered as locus to use for future monitoring of these hatchery samples.

TEESIER *et al.* (1997) have suggested that for Atlantic salmon the significant changes in allele frequencies between wild populations and hatchery fish

Table 4
Frequencies of estimates of null heterozygotes according to r_C *and* r_B *formulas and of observed null homozygotes risulting in no-bands at the three samples.*

	Pma3 Wildtype Hatchery			Pma4 Wildtype Hatchery			Pma5 Wildtype Hatchery		
	Japan	*Japan*	*Italy*	*Japan*	*Japan*	*Italy*	*Japan*	*Japan*	*Italy*
expected null homozygotes r_C	*0.01*	*0.03*	*0.02*	*0.13*	*0.29*	*0.02*	*0.01*	*0.003*	*0.005*
expected null homozygotes r_B	*0.008*	*0.02*	*0.01*	*0.07*	*0.11*	*0.01*	*0.008*	*0.002*	*0.005*
observed null homozygotes	*0.08*	*0.12*	-	*0.22*	*0.16*	*0.05*	*0.10*	*0.03*	*0.03*

are caused by genetic perturbations related to management practices. The loss of alleles is in fact more important than the change in allele frequencies, because the latter can be changed again in successive generations, also by genetic drift, while there is no way to recover a lost allele (ALLENDORF & PHELPS, 1980).

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