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Microsatellite polymorphisms in wild populations of European seabass: Preliminary results

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SUMMARY - Studies on population genetics of the European seabass (*Dicentrarchus labrax*) have shown the existence of extensive allozyme variation particularly between populations in different sea basins. The migration behaviour of juvenile and adult bass and the low levels of differentiation observed between samples in the same hydrographic area has lead to the assumption of a panmixia over quite large areas of the species range. Recently more detailed studies using allozymes, mtDNA and microsatellites and more powerful statistical methods are shedding new light on the population structure of this species. Microsatellites in particular because of their high levels of polymorphism are powerful markers for revealing population structuring. Five microsatellite loci were screened in over 300 individuals from 5 wild Portuguese populations. Preliminary results indicate high levels of polymorphism among the loci screened and the multilocus tests indicate the existence of population structuring at a small geographic scale.

Key words: Seabass, population genetics, Portugal, microsatellites.

RESUME *- "Polymorphismes microsatellites chez des populations sauvages de bar : Résultats préliminaires." Des études de génétique des populations chez le bar* (Dicentrarchus labrax) *ont montré une grande variation isoenzymatique, particulièrment entre populations de différents bassins marins. Le comportement migratoire des juvéniles et des adultes, et les bas niveaux de diffeérenciation observés dans la même aire hydrographique, ont permis de supposer l'existence de panmixie dans l'aire de distribution du bar. Récemment des travaux avec des allozymes, mtDNA et microsatellites en conjonction avec des méthodes statistiques plus puissantes en ont révélé plus sur la structure génétique des populations de cette espece. Les microsatellites sont des marqueurs hypervariables et grâce à eux on peut révéler la structure de populations séparées par de courtes distances géographiques. Cinq microsatellites chez plus de 300 individus de 5 populations de la côte portugaise on été étudiés. Les résultats préliminaires nous indiquent de hauts niveaux de polymorphisme chez les locus étudiés (de 20 à 41 allèles). Les tests multilocus montrent une différentiation génétique entre populations séparées par de courtes distances géographiques.*

Mots-clés *: Bar, loup, génétique des populations, Portugal, microsatellites.*

Introduction

The European seabass (*Dicentrarchus labrax*, L.) is a Serranidae found in the eastern Atlantic Ocean from Morocco to southern Norway and in the Mediterranean and Black Seas (Whitehead *et al.*, 1986). The reproduction of seabass occurs in marine coastal areas. Eggs and larvae drift towards estuaries and lagoons by passive movements, although larvae actively search for low salinity water. Young bass stay in these protected coastal areas for approximately two years. Juvenile

seabass engage in sporadic and occasional migrations which are often geographically restricted (Pickett and Pawson, 1994). Before reaching maturity, bass move offshore and can migrate long distances particularly in the northern part of the species range (Pickett and Pawson, 1994). What are thought to be spawning aggregations of seabass are often seen during February-March in Portuguese coastal areas. What is not known at this time is whether these are stable reproductively isolated sub-population maintained by some form of natal homing behaviour, or a random mix of individuals that happen to be in the area. Marine species with long pelagic and larval stages frequently show very low levels of populations differentiation, because there is often mixing of individuals between adjacent spawning areas due to the lack of clear geographic or hydrographic barriers. We wished to examine whether there is evidence for restricted gene flow and population differentiation in Portuguese populations of European seabass, in spite of this species' larval dispersal abilities and adult migrations.

It is a general view that more information on genetic aspects of the European seabass would contribute to enhance the management of both wild and captive populations. One of the major areas for recent research has been the development of effective microsatellite markers. The preliminary results presented here are a contribution in that area.

Population genetics

Published results on population genetics of the seabass used allozymes, mitochondrial DNA and microsatellite DNA and can be summarised as follows according to the type of genetic marker used.

Allozymes

Atlantic/Mediterranean Spanish populations - two genetically divergent groups, sharing no common polymorphic loci (Martínez *et al.*, 1991), Gulf of Biscay and Gulf of Cadiz, including Mar Menor. Genetic differentiation between French Atlantic and Mediterranean populations (Benharrat *et al.*,1984). Child (1992) suggests some level of reproductive isolation between Irish Sea and the English Channel, including the Thames Estuary. Allegrucci *et al.* (1994), however, found significant sub-structuring among Italian populations from the Tyrrhenian sea. There is evidence of genetic sub-population structure in a restricted geographical area, i.e. Portugal (Castilho and McAndrew, submitted).

MtDNA

Patarnello *et al.* (1993) using mtDNA sequence variation revealed frequency differences in haplotypes between an eastern group (North and South Adriatic Sea, Crete and East Sicily) and a western group (Tyrrhenian Sea and Sardinia) in the Mediterranean Sea.

Microsatellite DNA

Microsatellite markers suggest a slight genetic differentiation between Golfo-de-Valencia (Spain) and Gulf-du-Lion (France) (García de León *et al.*, 1997).

Material and methods

Three hundred and forty-one individuals were caught at five different locations along the Portuguese coast (Fig. 1).

A genomic library was created by partially digesting the DNA from a single seabass with *Sau3A* I. Fragments within the size range of 300-600 bp were selected. Ligation was performed between pUC 18 Bam HI/BAP (BRL Gibco) and the DNA fragments. Competent *E.coli* cells, strain DH5α (BRL Gibco) were used for the transformation. Replica nylon filters (Hybond-N-Amersham) were pre-hybridised (5xSSPE/5x Denhardt's/0.5% SDS/100 μg/ml RNA) for 1 hour at 65°C, prior to overnight hybridisation at 65°C to $(GT)_{15}$ synthetic oligonucleotides radio-labelled with [γ^{32}P]ATP and T4 Polynucleotide kinase. Filters were washed with 2xSSC/0.2%SDS at room temperature and with 0.2xSSC/0.2%SDS at 65°C. and were exposed to X-ray film (Kodak XAR-5) with two intensifying double screens at -80°C for 6 hours. Positive clones were picked off the plates and grown in LB-ampicillin for 48 hours. The plasmid DNA was extracted with phenol-chloroform and minipreparations and followed the classic alkaline lysis. Sequencing was performed with the T7 sequencing Kit, according to the manufactures instructions, using ^{35}S label. 8% acrylamide gels were run for 3.5 hours at 60 mA. The gel was fixed for an hour and then was dried in a Biorad gel dryer for 2 hours before being exposed overnight to Kodak X-omat R film.

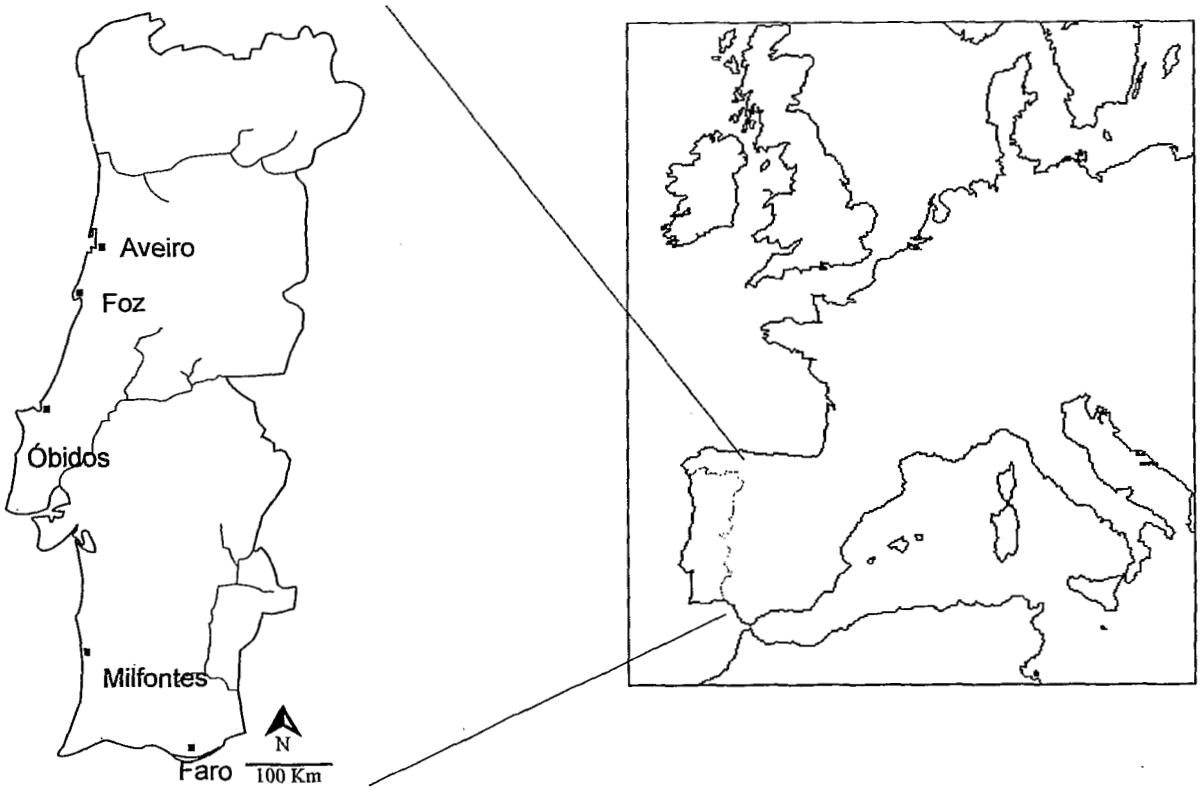

Fig. 1. Sampling locations.

PCR procedures followed Castilho and McAndrew (*in press*). All data were analysed with GENEPOP (Raymond and Rousset, 1995). *F*-statistics devised by Wright (1951) offer a means of summarising population structure. In this work heterozygote deficits attributable to among (F_{ST}) population components were estimated as θ (Weir and Cockerham, 1984, Weir, 1990).

Results

Two pairs of primers (Castilho and McAndrew, *in press*) were designed for microsatellites containing 10 or more repeat units, because below this size microsatellite *loci* tend to be less variable (Weber, 1990): *Dla6* and *Dla11* (GenBank/EMBL acc. nos Y13158 and Y13159). We also employed 3 sets of seabass microsatellite primers: *Labrax-3*, *Labrax-8* and *Labrax-9* previously developed by Garcia de León *et al.* (1997). Microsatellite repeat structure, range and number of alleles are presented in Table 1.

All five microsatellite loci surveyed were highly polymorphic in all seabass populations. The number of alleles observed per locus ranged from 20 for *Dla11* to 41 for *Labrax-3* (mean ± s.d. = 31.2 ± 8.4; Table 1). Observed heterozygosities were high, ranging from 0.45 for *Dla6* to 0.89 for *Labrax-3* (mean = 0.71)

Table 1. Structure (range in base numbers and smallest and longest PCR product), allele numbers and observed and expected heterozygosity per microsatellite locus for all samples pooled

Locus	Repeat structure/range	Allele numbers	Observed/ Expected heterozygosity
Dla6	$(AC)_{26}$ / 60:55-115	28	0.45/0.73
Dla11	$(GT)_{16}$ / 42:99-141	20	0.84/0.85
Labrax-3	$(GT)_{25}GC(GT)_7(AT)_3(GT)_3$ / 86: 116-202	41	0.89/0.92
Labrax-8	$(AC)_{19}$ / 58: 90-248	29	0.87/0.94
Labrax-9	$(AC)_{52}$ / 110:144-254	38	0.52/0.71
Multilocus			0.71/0.83

The allele frequency distributions were uni or multimodal, always exhibiting many rare alleles (Fig. 2). There were two major patterns that describe the allele frequency distribution of the microsatellite loci studied. *Dla11* showed one single mode, around allele 117. The other loci, show a number of modes separated in some cases by very similar number of repeats, namely *Labrax-3*, 18-20 repeats; *Labrax-8*, 14-18 repeats; *Labrax-9*, 20 repeats.

It is interesting to compare the distribution of *Labrax-3* and *Labrax-8* alleles

obtained in this study and those observed by García de León *et al.* (1997). The *Labrax-3* distribution is very similar, sharing the same most frequent alleles: 144, 150, 156, 162 and 182. The exceptions are the frequency of allele 120 (<5%) in France and a total absence of this allele in Portugal. The *Labrax-8* allele frequency distribution is quite different between Portuguese and French samples. While in Portugal no single allele has a frequency higher than 0.11 (allele 226) in France the most common allele has a frequency of nearly 0.45 (allele 210).

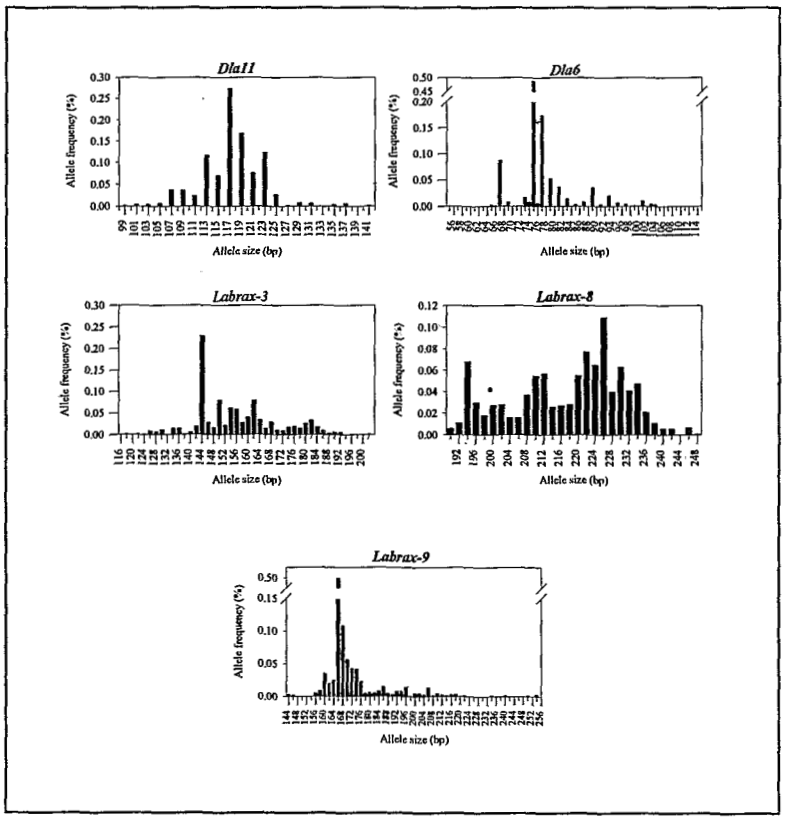

Fig. 2. Combined frequency histograms of allele sizes in base numbers in all the 5 populations.

The mean number of alleles was very high, ranging from 14.8 in Faro to 23.4 in Milfontes, however the expected heterozygosity values in all samples were very similar (Table 2).

When the population allelic differentiation test is carried out (Table 3), all loci except *Dla6* showed significant differences, indicating that the distribution of alleles is not identical across populations. Similarly, when a population genotypic differentiation test is carried out (Table 3), all loci apart from *Dla6* and *Labrax-3*, gave significant results, indicating that also the distribution of genotypes is not identical across populations. A combination of all tests, results in a global rejection of the null hypothesis for both allelic and genotypic cases, indicating the existence of population structuring.

Table 2. Sample sizes, mean alleles number ± standard deviation and observed (Ho) and expected (He) heterozygosity values (Nei, 1978)

Population	Sample size	Mean number of alleles per locus	Heterozygosity	
			Ho	He
Foz	75	20.0 ± 8.8	0.71 ± 0.25	0.83 ± 0.11
Aveiro	64	19.8 ± 6.0	0.69 ± 0.18	0.84 ± 0.09
Óbidos	74	22.0 ± 7.8	0.74 ± 0.21	0.84 ± 0.09
Milfontes	104	23.4 ± 9.9	0.74 ± 0.21	0.82 ± 0.11
Faro	24	14.8± 10.0	0.67 ± 0.30	0.81 ± 0.16
All populations	341	31.2 ± 8.4	0.71 ± 0.21	0.83 ± 0.10

Table 3. Fisher's exact test probability values of population allelic differentiation and G-likelihood test probability values for genotypic differentiation

Locus	Allelic differentiation		Genotypic differentiation	
	Probability	S.E.	θ	Probability
Dla11	0.00035*	0.00029	0.004	<0.0001*
Dla6	0.11950	0.02073	-0.002	0.3331
Labrax-3	0.00045*	0.00045	-0.001	0.1704
Labrax-8	0.00207*	0.00189	0.005	0.0017*
Labrax-9	0.00327*	0.00169	0.002	0.0029*
Multilocus	<0.0001*		0.0016	<0.0001*

*p<0.01 with Bonferroni adjustment for five simultaneous comparisons

Several works on population genetics of seabass using different types of markers, have pointed the existence of different levels of genetic differentiation: Atlantic/Mediterranean Spanish populations (Martínez *et al.*, 1991) and French Atlantic and Mediterranean populations (Benharrat *et al.*, 1984), geographically distant populations, show clear genetic differentiation. Populations geographically closer have also shown some degree of genetic differentiation: east (North and South Adriatic Sea, Crete and East Sicily)-west (Tyrrhenian Sea and Sardinia) Mediterranean populations (Patarnello *et al.*, 1993), Italian populations from the Tyrrhenian sea (Allegrucci *et al.*, 1994) and Golfo-de-Valencia (Spain) and Gulf -du-Lion (France) (García de León *et al.*, 1997). Our results can be considered as a

further indication that a marine species with high dispersal potential, can show genetic differentiation on small geographical scales.

The main conclusion that can be drawn from these preliminary results is that there is genetic differentiation among populations of seabass from the Portuguese coast (which is also temporally stable, data not shown). Further consideration will be given to testing neutrality of loci, analysing conformance to Hardy-Weinberg equilibrium, estimating effective sizes and population subdivision.

The high levels of polymorphism detected will provide a useful tool for the management of captive broodstock and selective breeding experiments. The non-invasive nature of the sampling because of the small quantity of tissue required for the PCR amplification of the microsatellites and the high levels of polymorphism observed, make microsatellites potentially highly informative markers particularly in pedigree breeding studies.

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