Isolation and characterization of nine polymorphic microsatellite markers in the two-banded sea bream (Diplodus vulgaris) and cross-species amplification in the white sea bream (Diplodus sargus) and the saddled bream (Oblada melanura)

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Abstract

We have developed nine new microsatellite markers for the two-banded sea bream (Diplodus vulgaris) from an enriched genome library protocol. All these loci are polymorphic, with mean allelic diversity of 13 (range 5–21), and expected and observed heterozygosities from 0.641 to 0.932 and 0.428 to 0.914, respectively. Cross-species tests in two close-related species of the genus Diplodus (D. sargus and O. melanura) revealed successful amplifications at 8 out of 9 loci, with mean allele number of 4.75 (range 2–8) and 5.50 (range 3–10), respectively. These results are consistent with the close phylogenetic relationships between the three species, indicating this set of primers might prove useful for studying the levels of genetic diversity and population differentiation in these three species and in other phylogenetically close species of the genus Diplodus and Sparus.

The family Sparidae is represented in the Mediterranean Sea by 11 genera and 24 species that usually inhabit coastal areas and produce pelagic eggs and larvae (Bauchot et al. 1986). Sea breams are commercially important species and have also recently gained considerable importance for aquaculture (Fischer et al. 1987). The two-banded sea bream, Diplodus vulgaris is a demersal species distributed in the Mediterranean and along the eastern Atlantic coast from France to Senegal (Bauchot & Hureau 1986). It can be found close to rocky and sandy bottoms, and juveniles often live in coastal lagoons and estuaries (Macpherson 1998). Although it is one of the most frequent and abundant sparid fish in the coastal waters of southern Europe, the amount of information available on several important aspects of its biology and on their stock assessment is very scarce. Furthermore, while several species of

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the same genus have been genetically extensively studied by means of allozymes and mitochondrial DNA for species differentiation (Reina et al. 1994; Alarcon & Alvarez 1999), phylogeography (Bargelloni et al. 2005) and genetic structure (Lenfant & Planes 1996, 2002; Lenfant 1998, 2003; Planes & Lenfant 2002; Ben Slimen et al. 2004; Gonzalez-Wanguemert et al. 2004; Perez-Ruzafa et al. 2006), genetic information is scarce for D. vulgaris. A single genetic work using allozymes markers revealed that D. vulgaris is genetically structured within the east Mediterranean basin (Arculeo et al. 2003), but more results are needed throughout its entire range. Marine species exhibit a great variability in population structure and genetic diversity patterns, and it is important to address specific questions for management purposes in commercially important species. Moreover, genetic populations' analyses aimed at identifying regional patterns of genetic connectivity between marine populations are critical for setting the appropriate geographical scales in which marine reserve systems will be best effective. In

Table 1 Characterization of eight two-banded sea bream (Diplodus vulgaris) microsatellite loci (N = 30). Locus name, repeat motif, fluorescent dye-′primer sequence, number of alleles, allele size range, H_O, observed heterozygosity; H_E, expected heterozygosity under Hardy–Weinberg equilibrium; F_{IS}, inbreeding coefficient, *P < 0.05

Locus	Repeat motif	Accession no.	Primer sequence (5′–3′)	No. of alleles	Allele size (bp)	H_O	H_E	F_{IS}	P values
Dvul11	$(CA)_{13}$	EF064289	F: VIC-GGCCCGCTTTATTCTCAGTCTCAA	5	135–147	0.428	0.641	0.309	0.075
			R: TGCAGGGAACAGAGGGATGACAG						
Dvul2	$(GT)_{28}$	EF064290	F: VIC-CTGCGTTGATGGTTTTTCAGAATG	11	180–206	0.571	0.843	0.319*	0.0062
			R: CTGCGAAACATCTGGAGTTGTATT						
Dvul33	$(CA)_{11}$	EF064291	F: FAM-GCCGGGCTCGACATTGACACTGAA	17	260–300	0.885	0.918	0.037	0.2625
			R: GCAGCCAGCAGAGCTTAAAGAACT						
Dvul4	$(CA)_{13}$	EF064292	F: NED-GCGGTTATGTATACGTTGCGTTTA	16	244–276	0.914	0.923	0.01	0.3625
			R: TTGGCGTTGAACAGAAGTCAGACA						
Dvul61	$(GT)_{18}GA(GT)_8$	EF064293	F: VIC-TGGGGACTCTCAGAATCATCACAA	7	412–426	0.838	0.689	−0.221	1
			R: TGGAAAAAGCCCTCTGGACAAAAG						
Dvul63	$(CA)_{14}$	EF064294	F: PET-GAGGAATGAGTAGAGAAAAGATGG	13	190–234	0.575	0.84	0.315*	0.0062
			R: ACCCCAACAACCAGAATACCTATA						
Dvul84	$(GT)_{15}$	EF064295	F: PET-GCTCGACGTGCACTCTGCCCTTGA	14	254–296	0.647	0.816	0.209	0.0125
			R: ATTCCCCAAATCCAGCACTCACAT						
Dvul38	$(CA)_{16}$	EF064296	F: FAM-TCGGGCACAGATAGAAAGAAACAC	21	170–210	0.914	0.932	0.016	0.4063
			R: GAAGGAAGACGGATCTCAGGATGA						
Dvul6	$(CA)_{14}$	EF064297	F: FAM-GGGCAAACAGGAGCAAAAAGCCAG	2§	419–425	NC	NC	NC	NC
			R: AGCCGCAGTTGATTTACAGAGTGT						

§, only two individuals were genotyped at this locus.

order to assess the genetic variability of D. vulgaris, we developed a set of dinucleotide microsatellites markers and test for their variability in two closely relative species, the white sea bream (Diplodus sargus) and the saddled bream (Oblada melanura).

Microsatellite markers were identified through the development of an enriched genomic library as described by Glenn et al. (2000). DNA extractions were performed from lateral fin tissue out of 10 D. vulgaris individuals from the western Mediterranean, and approximately 10 µg of high molecular weight DNA was isolated by phenol-chloroform extraction (Sambrook et al. 1989). Simultaneous restriction–ligation of genomic DNA was carried out using the RsaI restriction enzyme and double-stranded linker-adapted primers according to Hamilton et al. (1999). Ligated DNA was enriched by with a biotin-labelled probe mixture consisting of $(GT)_{10}$ and $(CT)_{10}$ at 10 µm each. DNA fragments with repetitive sequences were then selectively captured by streptavidin-coated Dynabeads (Oxoid) and separated by a magnetic field. Enriched DNA was eluted in 200 µL dH_2O from the bead probes and concentrated by vacuum centrifugation to a final concentration of ~100 ng/µL. Recovered DNA was then re-amplified by polymerase chain reaction (PCR), purified and ligated into a cloning vector using pGEM-T Easy Vector II (Promega). A total of 98 positive clones were screened and checked for inserts using ABI PRISM BigDye Terminator Cycle Kit (Applied Biosystems) and resolved on an ABI 3100 Genetic Analyser (Applied Biosystems). Primer pairs for nine

potentially usable microsatellite loci were designed using the software package oligo 6.4. Polymorphism was tested by multiplex PCRs performed in 20 µL total volume, which include 50 ng of DNA, 2 mm of $MgCl_2$, 0.25 µm of each primer, 200 µm dNTP's, 1× reaction buffer [75 mm Tris-Hcl, 20 mm $(NH_4)_2SO_4$] and 0.5 U Taq polymerase (BIOTAQ). Reaction conditions were as follows: an initial denaturation step of 5 min at 95 °C, eight cycles consisting of 45 s at 92 °C, 45 s at 53 °C annealing temperature, 45 s at 72 °C followed by an additional 24 cycles consisting of 30 s at 92 °C, 30 s at 55 °C annealing temperature, and 30 s at 72 °C. Microsatellite variability was assessed in 30 individuals from the western Mediterranean (Blanes). Individuals were genotyped by assessing allele size on an ABI 3100 Genetic Analyser (Applied Biosystems) using forward primers labelled with FAM (Sigma) and NED, PET and VIC (Applied Biosystems). Allele scoring was carried out using genemapper version software version 3.5 (Applied Biosystems). Expected and observed values for heterozygosity were determined using arlequin 2.0 (Schneider et al. 2000). The number of alleles per locus, allele size range as well as deviations from Hardy–Weinberg expectations and linkage disequilibrium between pairs of loci were estimated using fstat version 2.9 (Goudet 1995). All loci were polymorphic; the total numbers of alleles per locus and heterozygosity estimates are listed in Table 1. We found no evidence of linkage disequilibrium between locus pairs. Two loci showed significant departures from Hardy–Weinberg equilibrium (Dvul2, Dvul63) likely due

Table 2 Cross-species amplification of nine microsatellite loci from the two banded bream (Diplodus vulgaris) in the white sea bream (Diplodus sargus) and the saddled bream (Oblada melanura). Presented are locus name, number of alleles (N_a), allele size range; NA indicates non-amplification

Locus	D. sargus (n = 7)		O. melanura (n = 8)	
	N_a	Range	N_a	Range
Dvul11	2	144–146	3	144–150
Dvul2	3	182–186	4	182–192
Dvul33	8	282–302	10	274–304
Dvul4	8	248–280	7	248–268
Dvul61	6	390–440	6	404–450
Dvul63	NA	NA	NA	NA
Dvul84	7	254–274	7	256–284
Dvul38	2	170–174	4	170–176
Dvul6	2	345–349	3	341–349

to the presence of null alleles. Cross-species amplification was examined in two closed relatives (D. sargus and O. melanura) using the same conditions detailed for D. vulgaris. All except one locus (Dvul63) amplify in both species. All loci were polymorphic in both species, with allele number ranging from two to 10, depending on species and locus (see Table 2). This is consistent with the close phylogenetic relationships between the three species (De la Herran et al. 2001; Summerer et al. 2001; Day 2002). They provide a useful set of markers for studying the genetic diversity, population differentiation and for the genetic monitoring of farm populations of these three species, and might even proved useful in other phylogenetically close species of the genus Diplodus and Sparus.

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References

Alarcon JA, Alvarez MC (1999) Genetic identification of sparid species by isozyme markers: application to interspecific hybrids. Aquaculture, 173, 95–103.

Arculeo M, Lo Brutto S, Sirna-Terranova M, Maggio T, Cannizzaro L, Parrinello N (2003) The stock genetic structure of two Sparidae species, Diplodus vulgaris and Lithognathus mormyrus, in the Mediterranean Sea. Fisheries Research, 63, 339–347.

Bargelloni L, Alarcon JA, Alvarez MC et al. (2005) The Atlantic–Mediterranean transition: discordant genetic patterns in two sea bream species, Diplodus puntazzo (Cetti) and Diplodus sargus (L.). Molecular Phylogenetics and Evolution, 36, 523–535.

Bauchot ML, Hureau JC (1986) Sparidae. In: Fishes of the North-eastern Atlantic and the Mediterranean (eds Whitehead PJP, Bauchot ML, Hureau JC, Nielsen J, Tortonese E), Vol. II, pp. 883–907. UNESCO, Paris.

Ben Slimen H, Guerbej H, Ben Othmen A et al. (2004) Genetic differentiation between populations of gilthead seabream (Sparus aurata) along the Tunisian coast. Cybium, 28, 45–50.

Day JJ (2002) Phylogenetic relationships of the Sparidae (Teleostei: Percoidei) and implications for convergent trophic evolution. Biological Journal of the Linnean Society, 76, 269–301.

De la Herran R, Rejon CR, Rejon MR, Garridos-Ramos MA (2001) The molecular phylogeny of the Sparidae (Pisces, Perciformes) based on two satellite DNA families. Heredity, 87, 691–697.

Fischer W, Schneider M, Bauchot ML (1987) Fiches FAO d'identification des espèces pour les besoins de la pêche. Mediterranée et Mer Noire (Zone de Peche 37). FAO, Rome.

Glenn TC, Cary T, Dust M et al. (2000) Microsatellite isolation. http://www.uga.edu/srel/DNA_Laboratory/protocols.htm.

Gonzalez-Wanguemert M, Perez-Ruzafa A, Marcos C, Garcia-Charton JA (2004) Genetic differentiation of Diplodus sargus (Pisces: Sparidae) populations in the south-west Mediterranean. Biological Journal of the Linnean Society, 82, 249–261.

Goudet J (1995) fstat (2.9.3) a program for IBM-compatible PCs to calculate Weir and Cockerman's (1984) estimators of F statistics. Journal of Heredity, 86, 485–486.

Hamilton MB, Pincus EL, Di Fiore A, Flesher RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. BioTechniques, 27, 500–507.

Lenfant P (1998) Influence des paramètres démographiques sur la différenciation génétique intra- et inter-populations: le cas du poisson marin Diplodus sargus (Linné 1758). Doctoral Thesis, EPHE-University of Paris VI.

Lenfant P (2003) Demographic and genetic structures of white sea bream populations (Diplodus sargus, Linnaeus, 1758) inside and outside a Mediterranean marine reserve. Comptes Rendus Biologies, 326, 751–760.

Lenfant P, Planes S (1996) Genetic differentiation of white sea bream within the Lion's Gulf and the Ligurian Sea (Mediterranean sea). Journal of Fish Biology, 49, 613–621.

Lenfant P, Planes S (2002) Temporal genetic changes between cohorts in a natural population of a marine fish, Diplodus sargus. Biological Journal of the Linnean Society, 76, 9–20.

Macpherson E (1998) Ontogenic shifts in habitat use and aggregation in juvenile sparid fishes. Journal of Experimental Marine Biology and Ecology, 220, 127–150.

Perez-Ruzafa A, Gonzalez-Wanguemert M, Lenfant P, Marcos C, Garcia-Charton JA (2006) Effects of fishing protection on the genetic structure of fish populations. Biological Conservation, 129, 244–255.

Planes S, Lenfant P (2002) Temporal change in the genetic structure between and within cohorts of a marine fish, Diplodus sargus, induced by a large variance in individual reproductive success. Molecular Ecology, 11, 1515–1524.

Reina J, Martinez G, Amores A, Alvarez MC (1994) Interspecific genetic differentiation in Western Mediterranean sparid fish. Aquaculture, 125, 47–57.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schneider S, Roessli D, Excoffier L (2000) arlequin version 2.0: a software for population genetics data analysis. Genetics and Biometry Laboratory. University of Geneva, Switzerland.

Summerer M, Hanel R, Sturmbauer C (2001) Mitochondrial phylogeny and biogeographic affinities of sea breams of the genus Diplodus (Sparidae). Journal of Fish Biology, 59, 1638–1652.