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*)

S. ROQUES,* J. A. GALARZA,*† E. MACPHERSON,‡ G. F. TURNER† and C. RICO*

*Estación Biológica Doñana (CSIC), Avenue. Ma. Luisa S/N, 41013 Sevilla, Spain, †Department of Biological Sciences, University of Hull HU6 7RX, UK, ‡Centre d’Estudis Avançats de Blanes (CSIC), Carrer d’accés a la Cala Sant Francesc, núm. 14, 17300 Blanes, Catalunya, Spain

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*.

Keywords: *Diplodus*, microsatellite, *Oblada melanura*, sea bream, Sparidae

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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 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.
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\(_2\)O 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, 200 mM (NH\(_4\))\(_2\)SO\(_4\)] 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, Dvul6) likely due to the small sample size.
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 phylo-genetic 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


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.


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), allele size range; NA indicates non-amplification.

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