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Additional Information

1 **Assessment of parental contributions to fast- and slow-growing progenies in the sea bream *Sparus***
2 ***aurata* L. using a new multiplex PCR**

3

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16 *Keywords: Sparus aurata, microsatellite markers, multiplex PCR, OVIDORPLEX, parentage, inbreeding*

17

18 *Running title: Parentage study in Sparus aurata using a multiplex PCR*

19

20 **ABSTRACT**

21

22 Molecular tools to assist breeding programs in the gilthead sea bream (*Sparus aurata* L.) are scarce. A
23 new multiplex PCR technique (OVIDORPLEX), which amplifies nine known microsatellite markers, was
24 developed in this work. This multiplex system showed a high mean heterozygosity (>0.800) and a high
25 mean number of alleles per marker (>14) when tested in two sea bream broodstocks (A: 40 breeders and
26 B: 38 breeders). We tested this multiplex PCR for inferring parentage in a Spanish hatchery that graded
27 the animals by size as part of their management procedure. The progeny of the broodstock were divided
28 into fast- and slow-growth groups. Parentage studies revealed that this management procedure entailed a
29 global reduction of the breeders' representation in progeny and that breeders' contributions were
30 significantly unequal. Due to this, effective sample sizes fell to $N_e \approx 13-14$ for fast- and $N_e \approx 18-24$ for

31 slow-growth progeny groups. These results imply a 3 to 4% rate of inbreeding per generation in the fast-
32 growth group, which is more important to hatchery managers than the slow group. Not all the progeny
33 were evaluated in this experiment (due to the discarding steps), and thus it is difficult to know if the
34 phenotypic performance showed by the fast-growing progeny will be heritable. However, there were
35 genetic differences between the differentiated growth progeny groups (fast vs. slow, F_{ST} values=0.016 to
36 0.023; $P<0.01$). We also identified breeders with significantly different contributions to the fast- (10
37 breeders) or to the slow- (15 breeders) growth progeny groups. Our results demonstrated that this new
38 multiplex PCR could be useful for quantitative programs (breeding programs, detection of QTL,
39 inbreeding control or reconstruction of fish genealogies) to improve the aquaculture of the gilthead sea
40 bream (*S. aurata*).

41

42 **1. Introduction**

43

44 In the last decade, geneticists have helped aquaculture managers by implementing selection and breeding
45 programs (while preventing inbreeding) to obtain higher productivity and sustainability in fish hatcheries.
46 The use of molecular markers has significantly helped this goal. In particular, the use of microsatellites
47 has allowed the inference of effective breeding numbers (N_e) through parentage assignments in several
48 species, including Atlantic salmon (*Salmo salar*) (Norris et al., 2000), turbot (*Scophthalmus maximus*)
49 (Borrell et al., 2004), Atlantic cod (*Gadus morhua*) (Herlin et al., 2007, 2008), common dentex (*Dentex*
50 *dentex*) (Borrell et al., 2008), and European sea bass (*Dicentrarchus labrax*) (Bardon et al., 2009). This
51 approach allows pedigree reconstruction (indispensable for quantitative programs) and detection and
52 avoidance of inbreeding in hatcheries. Fish are highly fecund, and with only a few breeders it is possible
53 to obtain high numbers of eggs and sperm. However, high levels of inbreeding can cause a significant
54 decrease in growth rates or other production phenotypes (Kincaid, 1976, 1983; Gjerde et al., 1983;
55 Sbordoni et al., 1986; Su et al., 1996; Evans et al., 2004).

56

57 The gilthead sea bream (*Sparus aurata* L.) is a member of the family Sparidae and one of the most
58 important farmed fish in Europe (especially in the Mediterranean area). Its European production doubled
59 between 1999 [46,353 tons (t)] and 2006 (86,665 t). The major producers of this fish are Greece (56%),
60 Spain (24%) and Italy (10%) (Moretti et al., 1999; FEAP 2008). Although traditionally considered a

61 delicacy in the Mediterranean area, gilthead sea bream is now widely available in most parts of Europe.
62 This fish is a protandrous hermaphroditic, mass-spawning species in which individuals are males during
63 the first two years of life and then gradually become females. Members of the species breed once a year
64 during a six- to eight-week period (Zohar et al., 1978, 1995). Some animals either delay or never attain
65 sex reversal, possibly due to social, environmental, and/or genetic factors (Zohar et al., 1995). In its
66 natural environment, the gilthead sea bream spawns between October and January, but spawning occurs
67 later in the eastern Mediterranean, where mean water temperatures are higher. Spawning in the wild
68 occurs in large groups or schools. A minimum of five to seven fish appears to be necessary to reduce
69 stress and induce natural spawning in artificial environments (Brown 2003 and references therein).

70

71 There is a lack of efficient breeding programs for the gilthead sea bream. Although estimates of genetic
72 variance, heritability, and phenotypic and genetic correlations between traits are now becoming available
73 for this species (e.g., Navarro et al., 2009), breeding programs require funding and infrastructure, both of
74 which are limited. In the most recent and complete study yet, Navarro et al. (2009) found low to medium
75 heritability estimates in gilthead sea bream at harvest (509 days) for body weight, fork length, condition
76 factor, gutted body weight, fillet weight, dressing percentage, and fillet percentage. Other authors have
77 found significant heritabilities for these and other traits (Knibb et al., 1997, 2000; Batargias, 1998;
78 Afonso et al., 2000; Thorland et al., 2007). These data on heritability have stimulated the gilthead sea
79 bream industry to improve production through the exploitation of additive genetic variation.

80

81 Useful molecular tools (e.g., efficient multiplex PCRs and typing of SNPs and QTL) to assist breeding
82 programs in gilthead sea bream aquaculture are still scarce. Franch et al. (2006) reported the first linkage
83 map for the gilthead sea bream using 204 previously known and novel microsatellites, and Cenadelli et al.
84 (2007) identified 76 SNPs in this species. The use of microsatellites in multiplex PCR has great value
85 allowing geneticists to reduce the cost per reaction and optimize analyses of genealogy reconstruction and
86 genetic variability within a population (Navarro et al., 2008). Navarro et al. (2008) developed the first two
87 microsatellite multiplex systems to infer kinships in the gilthead sea bream, and the first QTL for
88 resistance to fish pasteurellosis recently were reported by Massault et al. (2010).

89

90 In light of these circumstances, we designed a multiplex PCR system including nine previously known
91 microsatellites (from gilthead sea bream and related species) that show high levels of genetic variation
92 (Borrell et al., 2007). Our goal was a quick, inexpensive and efficient genetic analysis that could be useful
93 for quantitative programs in the culture of this species (breeding programs, detection of QTL, inbreeding
94 control or reconstruction of fish genealogies). We tested the multiplex PCR system in a Spanish
95 Mediterranean hatchery (Piscicultura Marina Mediterránea, S.L. Burriana, Castellón, Spain). This
96 hatchery possessed two gilthead sea bream broodstocks (group A: 40 breeders and group B: 38 breeders)
97 that were established in 2006 using a combinatorial optimization approach for guaranteeing the maximum
98 amount of genetic variation, which results in low inbreeding values in future matings (Borrell et al.,
99 2007). Untagged progeny from a single day of spawning ($n=520$ fish) from these two sea bream
100 broodstocks were raised to 18 months of age with two successive steps of phenotypic selection for size (to
101 obtain fast- and slow-growth progeny groups). Fast-growth, which is intrinsically related to better food
102 conversion, is an important trait for the sea bream industry; feeding represents approximately 40% of the
103 production cost for gilthead sea bream (Jover, 2007). Fast-growth is also correlated with disease
104 resistance or survival in several fish species (Gjedrem and Olesen, 2005) and shows fairly high
105 heritability ($h^2=0.2$ to 0.4) and considerably high percentages of genetic gain per generation (Gjedrem and
106 Thodesen, 2005). The size-based grading system used by the hatchery is common when rearing fish in
107 communal conditions to avoid negative social interactions such as competition for access to food.
108 However, this kind of management could potentially introduce bias in estimates of genetic parameters
109 because tank effects might be confounded with genetic potential (Blonk et al., 2010).

110

111 As a result of the management procedure discussed above, the hatchery had progeny from both
112 broodstocks showing fast- and slow-growth rates during culture. This system of fish classification is
113 based only on phenotypic values and includes several progeny discarding steps, which makes it difficult
114 to estimate additive genetic components. However, genetic analysis of these samples using the new
115 multiplex PCR technique OVIDORPLEX and a parentage study could help to answer relevant questions:
116 Are there quantitative or qualitative genetic differences between the differentiated growth rate progeny?
117 Are all the breeders contributing equally to the progeny? Are there breeders showing a significant
118 differential parental contribution to the differentiated progeny growth groups examined here?

119

120 2. Materials and methods

121

122 2.1 Fish

123 From a total of 101 *Sparus aurata* adult individuals (80 females and 21 males) that were available in
124 December of 2006 in a hatchery in Spain (Piscicultura Marina Mediterránea, S.L. Burriana, Castellón,
125 Spain), two broodstocks (Broodstock A: 29 females, average weight $1998.2 \pm$ (standard error) 43.5 g and
126 11 males, average weight 1877.2 ± 83.4 g; Broodstock B: 28 females, average weight 1992.8 ± 50.1 g,
127 and 10 males, average weight 1950.0 ± 76.3 g) were grouped using a combinatorial optimization
128 approach that followed the procedures described in Borrell et al. (2007). Briefly, the best possible
129 aggregations of approximately 40 breeders per broodstock were selected, taking into account the
130 relatedness coefficients (Queller and Goodnight, 1989) among the breeders. Values of the Queller and
131 Goodnight's relatedness coefficient range from -1 to +1. Theoretically, full-sib individuals share, on
132 average, 50% of their genome constitution and hence the relatedness coefficient (r) among them should
133 be near 0.5, while for pairs of half-sibs, it is expected that $r \approx 0.25$ and among pairs of unrelated
134 individuals $r \approx 0$. A positive value of relatedness would therefore indicate greater than expected
135 relatedness, and a negative value would suggest that two individuals are more divergent than expected
136 (Queller and Goodnight, 1989). Relatedness coefficients ranged from $-0.39 < r < +0.83$ in the complete
137 stock and were restricted to $-0.28 < r < +0.54$ in Broodstock A and $-0.25 < r < +0.43$ in Broodstock B.

138

139 The breeders within each tank were allowed to spawn freely in March of 2007. Fertilized eggs coming
140 from a single day were collected and incubated under identical conditions in two tanks until hatching.
141 This process was carried out on different days for the two different broodstocks, but the harvest was
142 always in the middle of the spawning period because it has been suggested that majority of the breeders
143 are contributing to descendants at that time (C. García-Fernández, Univ. of Oviedo, unpublished). All
144 larvae were reared in isolated large tanks until June of 2007, 86 and 78 days post-hatching for batches A
145 and B, respectively. At this point, untagged individuals were separated in fast- and slow-growth groups
146 according to their body width distributions using different sieves. In batch A, fish with a body width
147 greater than 4.5 mm (fast) or smaller than 3.5 mm (slow), were separated, whereas the batch B fish with a
148 body width higher than 3.5 mm or smaller than 2.5 mm formed fast- and slow-growth groups,
149 respectively.

150

151 The fish were moved to Universidad Politécnica de Valencia (Valencia, Spain) at the age of 165 and 157
152 days post-hatching, respectively, for batches A and B. They were released into 1,750-l fiberglass tanks,
153 where they were reared under intensive conditions. The tanks were in a recirculating marine water system
154 (30-m³ capacity) with a rotary mechanical filter and a gravity bio-filter. All tanks were aerated, and the
155 water temperature was maintained at $22.2 \pm 2.7^{\circ}\text{C}$ by a heat pump that was installed in the system. The
156 photoperiod was natural, and all tanks had similar light conditions. Fish were fed using commercial fish
157 feed (Dibaq S.A., Segovia, Spain) by hand twice a day until apparent satiation. At an intermediate stage
158 of growth (311 and 303 days post-hatching for batches A and B, respectively), 600 juveniles were
159 separated by weight into four groups: A12-Fast (A12F>41g), A12-Slow (A12S<15g), B12-Fast
160 (B12F>31g) and B12-Slow (B12S<12g). The fish were placed in four 4,000-l tanks and reared under
161 intensive conditions to commercial size.

162

163 Around 540 fish ($n=150$ for A18F, $n=150$ for A18S, $n=120$ for B18F, and $n=120$ for B18S) were weighed
164 at 528 and 520 days post-hatching for batches A and B, respectively. A small piece of the caudal fin was
165 preserved in 96% ethanol for genetic analysis. Mean weight differences among groups were evaluated
166 using the Kolmogorov-Smirnov test (a non-parametric test using two independent samples and the Z
167 statistic), available in the SPSS 15.0 statistic software.

168

169 *2.2 Microsatellite amplification and scoring*

170 Genomic DNA was purified from a small piece of the caudal fin using *Chelex*^R 100 (Walsh et al., 1991).
171 We developed a novel multiplex PCR (OVIDORPLEX) using the Multiplex Manager 1.0 software
172 (Holleley and Geerts, 2009) and the gilthead sea bream microsatellite data previously collected from more
173 than 800 gilthead sea bream individuals (Borrell et al., 2007) (Figure 1). OVIDORPLEX included nine
174 microsatellite loci and fluorescent dyes: *VIC-SaGT1* (0.6 μM), *VIC-SaGT26* (0.3 μM), and *NED-*
175 *SaGT41b* (0.5 μM) (Batargias et al., 1999); *VIC-Pb-OVI-D106* (0.6 μM), and *PET-Pb-OVI-D102* (0.4
176 μM) (Piñera et al., 2006); *FAM-Dxd44* (0.4 μM), and *FAM-Dpt3* (0.4 μM) (De la Herrán et al., 2005) and
177 *NED- μ l84* (0.3 μM), and *PET- μ l90* (0.3 μM) microsatellites (Power et al., 2003). The QIAGEN
178 multiplex PCR kit protocol with an annealing temperature of 52°C was used to obtain 15 μl of the

179 OVIDORPLEX amplifications. Parental and offspring genotypes were scored after the analysis of the
180 amplification products on the ABI 3130XL Genetic Analyzer using Genemapper 4.0.

181

182 *2.3 Genetic diversity analysis*

183 The number of alleles at each microsatellite locus (N_A), the percentage of polymorphic loci ($P_{0.95}$), the
184 proportion of individual samples that were heterozygous (direct count heterozygosity, H_o) and the
185 unbiased estimate of heterozygosity (H_e) for each group were assessed using Cervus 3.0 (Marshall et al.,
186 1998). The Fstat statistical package (2.93 version) (Goudet, 1995, 2001) was used to estimate the total
187 variation in gene frequencies (F_{IT}), partitioned into components of variation occurring within (F_{IS}) and
188 among (F_{ST}) samples for each locus following Weir and Cockerham (1984). Significance levels of F_{IS}
189 were assessed through randomization of alleles (1,000 times) within samples (Fstat) for each broodstock.
190 Pairwise F_{ST} values between samples and P -values were calculated using Fstat (for significance levels of
191 F_{ST} , multi-locus genotypes were randomized between pairs of samples (1000 permutations), and then the
192 significance after strict Bonferroni correction was calculated (Rice, 1989; Goudet, 2001)).

193

194 *2.4 Parentage assignments*

195 We used the Cervus 3.0 software (Marshall et al., 1998; Kalinowski et al., 2007) to assign parentage. This
196 program calculates both the *a priori* polymorphic information content (PIC) for every locus from each
197 broodstock and the total exclusionary power (E). In addition, the program simulates parental assignments.
198 The total exclusionary power is defined as the probability of excluding an arbitrary, unrelated parent
199 candidate. When multiple parent candidates are not excluded, the exclusionary approach is inadequate
200 (Cervus 3.0, Marshall et al., 1998). The parentage assignment simulations were carried out, taking into
201 account the number of breeders per broodstock. Ten thousand cycles of simulated assignments were
202 carried out using 95% confidence intervals. Finally, after genotyping, all the offspring were assigned to
203 the most likely candidate parent pair with sexes known. In the assignment procedures, we allowed for
204 typing errors (0.05), as this dramatically reduces the impact of two other possible causes of mismatches in
205 parent-offspring relationships: mutations and null alleles (Marshall et al., 1998).

206

207 *2.5 Estimating effective breeding numbers (N_e)*

208 The rate of inbreeding (ΔF) and the effective population size (N_e) are related as $\Delta F = 1/2 N_e$ (Falconer,
209 1989). We first used the classical formula for estimating N_e : $N_e = 4 (Nm \times Nf) / (Nm + Nf)$ where $Nm =$
210 the number of male breeders and $Nf =$ the number of female breeders (Falconer, 1989). We also used an
211 approach previously assessed by Brown et al. (2005) and reassessed by J. Woolliams (Roslin Institute,
212 pers. comm.). This approach takes into account the proportion of descendants left by “presumed”
213 unrelated parents to the next generation following Hill (1979) and Woolliams and Bjima (2000);
214 Woolliams proposed $\Delta F = \sum c_{i(m)}^2 / 8 + 1/(32m) + \sum c_{i(f)}^2 / 8 + 1/(32f)$; with c_i being the fractional
215 contribution of males ($c_{i(m)}$) and females ($c_{i(f)}$) to offspring and m and f the number of males and
216 females, respectively. This formula is Woolliams’s derivation from the Hill (1979) formulation in terms
217 of offspring contributions, and has no selection component. It simplifies to Wright’s formula for Poisson
218 family sizes and, even when it is needed to be treated with caution, it is a useful approximation to the
219 problem of estimating N_e when unequal breeders contributions to offspring occur (J. Woolliams, Roslin
220 Institute, pers. comm.; Borrell et al., 2008).

221

222 *2.6 Differential parental contribution to progenies*

223 Contingency tables were used to (a) assess whether breeders contributed equally to progenies in a global
224 sense and also inside the different growth groups in each broodstock, and (b) evaluate different
225 contributions of male and female breeders to either the fast- or slow-growth groups. For the last test, a re-
226 sampling method (Patefield, 1981; Corral, 2005; Carleos, 2010) was used with the contingency table
227 frequencies. Under the null hypothesis of equal contribution and homogeneity, 100,000 contingency
228 tables were pseudo-randomly generated, while keeping the marginal totals constant (Patefield, 1981). For
229 each breeder, the proportion of tables where the frequency in the fast-growth group is more extreme than
230 or so extreme as in the observed contingency table was recorded. Slow-growth group frequencies were
231 handled similarly. This procedure quantifies how likely it is to obtain the observed frequencies (or more
232 extreme frequencies) for the corresponding breeder under the null hypothesis. Thus, low values (less than
233 0.05) indicate unlikely frequencies under the null hypothesis. The entire re-sampling procedure was
234 carried out in the R statistical environment (R Development Core Team, 2009).

235

236 **3. Results**

237

238 3.1 The OVIDORPLEX and the genetic characterization of broodstocks and differentiated growth
239 progeny groups

240 In this work, progeny from two *Sparus aurata* broodstocks were separated twice by size in a hatchery.
241 Samples were taken at 18 months of development, a point at which we have observed significant weight
242 differentiation between fast- and slow-growth groups in both broodstocks (**A** - A18F: 295.4 ± 4.4 g,
243 A18S: 195.7 ± 2.5 g, Z weight $_{A18F-A18S}=6.83$, $P<0.001$; **B** - B18F: 323.9 ± 4.8 g, B18S: 184.1 ± 2.9 g, Z
244 weight $_{B18F-B18S}=6.91$, $P<0.001$) (Table 1). We found significant weight differences between
245 corresponding size classes of A and B progeny groups (Z weight $_{A18F-B18F}=4.42$, $P<0.001$; Z weight $_{A18S-}$
246 $_{B18S}=3.28$, $P<0.01$).

247
248 Almost all of the individuals (516/520 offspring and the 78 breeders) were successfully genotyped at nine
249 microsatellite loci using OVIDORPLEX (Figure 1). The system was reliable even with the quick and
250 inexpensive Chelex DNA extraction method that results in low quality DNA. We detected three small
251 artifact peaks (channels of VIC-165bps, NED-167bps and 6-FAM-199bps), but they did not complicate
252 the genotyping process (Figure 1).

253
254 The genetic characteristics of all the groups analyzed are presented in Table 1. Breeders showed high
255 levels of genetic variation ($N_A > 14$ and $H_O > 0.800$). We also found agreement with the Hardy-Weinberg
256 expectations for populations under equilibrium (non-significant F_{IS} values). Both breeder groups were
257 found to be genetically similar ($F_{ST} = -0.005$ $P=0.996$). In terms of quantitative and/or qualitative genetic
258 differences between the fast- and slow-growth groups in the two analyzed broodstocks, we did not
259 observe heterozygosity level differences between them (Table 1). At 18 months of development, all
260 samples showed significant H-W disequilibrium due to an excess of heterozygotes. This excess was more
261 significant in the fast- ($P<0.001$) than the slow-growth progeny ($P<0.05$) (Table 1). Fast- and slow-
262 growth progeny were significantly differentiated within broodstocks ($F_{ST A18F-A18S} = 0.023$, $P=0.003$; F_{ST}
263 $_{B18F-B18S} = 0.016$, $P=0.003$). Corresponding fast- or slow-growth groups were genetically distinct between
264 broodstocks ($F_{ST A18F-B18F} = 0.043$, $P=0.003$; $F_{ST A18S-B18S} = 0.036$, $P=0.003$)

265

266 3.2 Parentage assignments and effective breeding number (N_e) estimations

267 The parentage assignment procedures carried out here revealed that the true parental pair was expected,
268 by simulation, to be found 99.9% of the time using the multiplex PCR including nine microsatellite loci
269 (Table 1). Almost all the progeny (516 of 520 collected after 18 months of development, 99.2%) were
270 assigned to a parental pair with certainty. In the A broodstock, 11 breeders did not produce progeny
271 (27.5%: 10 ♀ and 1 ♂), while in the B broodstock, 12 breeders failed to contribute to offspring (31.5%:
272 11 ♀ and 1 ♂).

273

274 The effective breeding numbers, taking into account the numbers of all males and females in the tank,
275 would be $N_e = 31$ and $N_e = 29$ in the A and B broodstocks, respectively. However, not all the breeders
276 contributed to offspring, and this implied reductions in the N_e estimates (A18F: $N_e \approx 18$, A18S: $N_e \approx 26$;
277 B18F: $N_e \approx 20$, and B18S: $N_e \approx 22$) (Table 1). We observed a globally significantly unequal contribution of
278 breeders to progeny in both broodstocks (**A**: 19 females, 14.6 descendants expected for each female
279 assuming equal contributions, $\chi^2 = 356.3$, $P < 0.001$; 10 males, 27.8 descendants expected for each male if
280 there are equal contributions, $\chi^2 = 221.6$, $P < 0.001$) (**B**: 17 females, 14.0 descendants are expected for each
281 female assuming equal contributions, $\chi^2 = 281.1$, $P < 0.001$; 9 males, 26.4 descendants expected by each
282 male if contributions are equal, $\chi^2 = 230.9$, $P < 0.001$). A similar result was found within growth groups.
283 Unequal reproductive success affected N_e estimates. The “global” effective breeding numbers calculated
284 here using Woolliams’s approach revealed significantly lower N_e values than expected with equal
285 contributions, primarily in the fast-growth groups (A18F: $N_e \approx 13$, A18S: $N_e \approx 24$; B18F: $N_e \approx 14$, and B18S:
286 $N_e \approx 18$) (Table 1).

287

288 *3.3 Differential parental contributions to fast- and slow-growth progeny groups*

289 The breeders did not contribute equally to fast- and slow-growth progeny in this experiment (**A**: females
290 $\chi^2 = 114.2$, $P < 0.001$; males $\chi^2 = 69.2$, $P < 0.001$) (**B**: females $\chi^2 = 84.3$, $P < 0.001$; males $\chi^2 = 50.6$, P
291 $P < 0.001$). This observation means that we found differential parental contributions to the growth
292 performance groups under study in both broodstocks (Figure 2).

293

294 The individual breeder’s χ^2 values were analyzed using a re-sampling method to identify the breeders
295 responsible for differential contribution to the fast- or slow-growth progeny groups. At 18 months of age,
296 eight out of 19 females and six out of 10 males left descendants at a differential rate in fast- and slow-

297 growth groups in the A broodstock (14/29=48%, 14 breeders left 76% of the descendants), while seven
298 out of 17 females and four out of nine males were identified in the B broodstock (11/26=42%, 11 breeders
299 were responsible for 74% of the descendants) (Figure 2). To summarize, we identified 10 breeders (six
300 females and four males) that contributed significantly more to fast-growth progeny and 15 that
301 contributed significantly more to slow-growth progeny; 30 breeders left descendants in equal proportions
302 to the two growth groups under study, while 23 breeders had null contributions in this experiment.

303

304 **4. Discussion**

305

306 *4.1 The multiplex PCR system*

307 The multiplex PCR developed in this work (OVIDORPLEX) was highly effective using our samples, and
308 it was less costly in terms of both time and money than scoring each marker with independent PCRs and
309 gel electrophoreses. This multiplex system showed high mean heterozygosity (>0.800) and high mean
310 number of alleles per marker (>14) when tested in two gilthead sea bream broodstocks (A: 40 breeders
311 and B: 38 breeders). These variability values are higher than those reported for the Rim-A and Rim-B
312 multiplex PCR by Navarro et al. (2008) and allow highly accurate determination of parentage, which is
313 important for correctly reconstructing fish genealogies and for estimating genetic parameters in breeding
314 programs.

315

316 It is possible to adjust OVIDORPLEX for use with high-quality DNA (e.g., that produced by the Qiagen
317 DNA extraction kit). This adjustment entails the use of lower amounts of PCR primers and/or raising the
318 annealing temperature (OVIDORPLEX works well in the 52 to 55°C range), which should produce even
319 better results by doubling the intensities of the microsatellite signals and diminishing the signals from
320 artifact peaks. Amplification failures of any of the loci used in OVIDORPLEX would not significantly
321 affect parentage studies. The use of OVIDORPLEX-R (with R standing for reduced) using only five of
322 the nine microsatellites included in OVIDORPLEX (*Dxd44*, *Dpt3*, *SaGT41b*, *SaGT2*, and *Pb-OVI-D106*)
323 in parentage assignment simulations resulted in approximately 95% of parent pairs being correctly
324 identified in our two broodstocks. However, simulated data are not real data. Null alleles or degraded
325 tissues that result in low PCR amplification or failure to amplify some alleles can affect genotyping and
326 parentage assignments (Borrell et al., 2004).

327

328 *4.2 The breeding aspects*

329 The hatchery Piscicultura Marina Mediterránea, S.L. (Burriana, Castellón, Spain) uses a size-based
330 grading system for rearing fish. As a result of this system, the hatchery has progeny from two broodstocks
331 potentially showing fast- and slow-growth rates during culture. We used the OVIDORPLEX system for
332 performing parentage studies, which revealed that the size-based hatchery management procedure
333 produces a significant reduction in the breeder's participation in the maintenance of the A and B
334 broodstocks, and significantly unequal breeders' contributions to progeny. This outcome seriously
335 affected the effective breeding number estimates. Effective sample sizes fell to $N_e \approx 13-14$ for fast-
336 (representing only 32 to 36% of all the breeders involved) and $N_e \approx 24$ (60%)/ $N_e \approx 18$ (47%) for slow-
337 growth progeny groups in the A and B broodstocks, respectively. Our results imply a 3 to 4% rate of
338 inbreeding in the fast-growth groups, which are more important to hatchery managers than the slow-
339 growth groups. These inbreeding levels are similar to those previously found by Brown et al. (2005) in
340 this species without a selection strategy, although they used a higher number of breeders per broodstock
341 ($n=48$ to 58; Brown et al., 2005). Probably, the initial broodstock composition (maximized for genetic
342 variation in our case) had positively influenced the accumulation of inbreeding to levels comparable to
343 those from Brown et al.'s study (2005), even when two steps of phenotypic selection were performed
344 here. As a general rule, there should be only a 0.5% of rate of inbreeding per generation (ΔF) in breeding
345 schemes (Sonesson et al., 2005). Tave (1999) has suggested that managers from small, medium and large
346 hatcheries wanting minimum risk should avoid 5% of inbreeding accumulation over generations, although
347 this would require more management effort. More than a 12% accumulation of inbreeding in rainbow
348 trout allows inbreeding depression of valuable fitness traits (Gjerde et al., 1983; Fjalestad, 2005). A rate
349 of 5% of accumulated inbreeding was almost reached in this study in only one generation. If the progeny
350 showing fast-growth are to be used as breeders in upcoming culture cycles, some strategies should be
351 considered to diminish inbreeding. To introduce wild breeders into the culture cycles is a common
352 procedure, although this approach affects not only rates of inbreeding but probably also genetic gain,
353 which will likely be reduced. One plausible remedy could be rotational line crossing (Kincaid, 1977;
354 Tave, 1999) using genetically unrelated fast-growth progeny from the two broodstocks (the pedigree is
355 now available). Cohorts can be quite useful for preventing inbreeding over four to six generations and
356 minimizing inbreeding for several generations thereafter (Tave, 1999).

357

358 In addition to the challenge of avoiding inbreeding accumulation for upcoming culture cycles using this
359 type of hatchery management strategy, the prevention of environmental sources of variation in growth
360 from becoming confounded with heritable differences during selection is a serious concern. The most
361 problematic issue is that not all the progeny were evaluated in this experiment (due to the discarding
362 steps), and it is thus difficult to know whether the phenotypic performance showed by the fast-growing
363 progeny would be heritable. In any case, the genetic characterization of the samples and the parentage
364 study carried out here could yield insights into it as suggested below.

365

366 The genetic assessment of the broodstocks and their progeny (using nine randomly chosen microsatellite
367 markers) revealed H-W equilibrium for the two broodstocks under study. However, significant excesses
368 in heterozygosity were found in 18-month-old progeny; this is a characteristic usually seen in recently
369 bottlenecked populations (Luikart and Cornuet, 1997) (it also may be attributable to heterozygotes
370 advantage or selection acting against inbred (i.e., overly homozygous) individuals). More relevantly, fast-
371 and slow-growth groups were genetically different in both broodstocks (F_{ST} values=0.016 to 0.023;
372 $P<0.01$). It has been suggested that a correlation between Q_{ST} values (a measure of differentiation in
373 quantitative loci) and F_{ST} values is generally expected (Merila and Crnokrak, 2001), which implies that
374 molecular markers may be used as an index of the degree of differentiation at quantitative loci (Roff,
375 2003; Leinonen et al., 2008). Thus, genetic differences found between the fast- and slow-growth groups
376 in these two broodstocks' progeny using neutral molecular markers also suggest differentiation at QTL.
377 Moreover, we identified breeders with significantly different contributions to fast- (10 breeders) and to
378 slow- (15 breeders) growth groups. The rest of the breeders (67%) either did not contribute to progeny or
379 contributed in an equal manner to both growth groups. These findings could lead us to expect that a
380 genetic component is guiding the growth performance observed here (fast-growth often shows high
381 heritability in fish; $h^2=0.2$ to 0.4 ; Gjedrem and Thodesen, 2005). However, our results have an implicit
382 sampling effect (as a consequence of using only one-day spawning eggs, the posterior discarding steps, or
383 even the sampling for genetic analyses). A more complete design should take into account that the
384 spawning dynamics in *Sparus aurata* seem to be sequential (unpublished results). Thus, a mix of eggs
385 from several days and several points during the spawning season would be the best choice for maintaining
386 as much of the genetic variation of the parental stock as possible in the progeny. Preserving samples from

387 all the phenotypic classes will help to correctly evaluate the breeders' values for fast-growth, also
388 allowing an estimation of additive genetic components. Sonesson (2003) suggested that it is possible to
389 raise the selection intensity in multi-spawners such as the gilthead sea bream using progeny testing. This
390 increased selection intensity could help save space and money during culture by preserving only the
391 valuable breeders for upcoming culture cycles.

392

393 In summary, this work demonstrated that a new multiplex PCR technique, OVIDORPLEX, is a quick and
394 inexpensive method of genetic analysis that allows parentage studies that provide information about
395 breeders' contributions. This information can be used to assess the population's N_e , which provides a
396 good estimate of inbreeding, a process that should be controlled in hatcheries performing any kind of
397 management strategies. Parentage studies using microsatellite loci also facilitate the identification of
398 breeders that contribute significantly to the phenotypes of interest and allow an efficient genetic
399 characterization of the samples. The findings of this study should be relevant for the establishment of
400 successful breeding programs in aquaculture of the gilthead sea bream.

401

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413

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Table 1. Growth, genetic variability parameters and effective breeding numbers ($N\hat{e}$) estimations in *S. aurata* L. after genetic analysis using nine microsatellites included in the multiplex PCR OVIDORPLEX.

<u>Broodstock</u>	<u>A</u>			<u>B</u>		
	P	<i>Fast</i>	<i>Slow</i>	P	<i>Fast</i>	<i>Slow</i>
Collection day (approx. months)	-	528 18	528 18	-	520 18	520 18
<i>n</i>	40	139	139	38	118	120
Mean Weight ^Δ (g)	1965.0	295.4	195.7	1981.5	323.8	184.1
St. dev.	249.4	52.3	29.5	256.6	52.7	32.4
St. error	39.4	4.4	2.5	41.6	4.8	2.9
N_a	14.6	12.2	14.0	14.0	12.2	11.8
H_e	0.867	0.801	0.849	0.872	0.832	0.842
H_o	0.883	0.867	0.865	0.858	0.893	0.859
F_{IS} (<i>p values</i>)	-0.028 n.s (0.0843)	-0.085 *** (0.0009)	-0.019 * (0.0407)	+0.011 n.s (0.7300)	-0.073 *** (0.0009)	-0.022 * (0.0315)
<i>Sim</i>	99.9%	-	-	99.9%	-	-
<i>Breeders</i> [□]	29♀x11♂	11♀x8♂	19♀x10♂	28♀x10♂	14♀x8♂	14♀x9♂
$N\hat{e}$ (1)	31.2	18.5	26.2	29.4	20.3	21.9
$\Delta F(1)$ (%)	1.60	2.70	1.90	1.70	2.46	2.28
$N\hat{e}$ (2)	-	12.7	24.6	-	14.2	17.6
$\Delta F(2)$ (%)	-	3.91	2.02	-	3.50	2.83

P: Parental breeders. *n*: Number of individuals. Mean weight ^Δ: At December 2006 for breeders and at day of collection for progenies. St. dev.: Standard deviation. St. error: standard error. N_a : Number of alleles per locus; H_e : Expected Heterozygosity; H_o : Observed Heterozygosity. F_{IS} : Degree of departure from expected Hardy-Weinberg proportions within groups. *Sim*: Percentages of times that the true parents pair was found after ten thousand cycles of simulated assignments using 95% confidence intervals. *Breeders* [□]: All the possible breeders among parents, breeders that left progenies after parental assignments assessments using microsatellites in progenies. $N\hat{e}(1)$: Effective population sizes using $4x(Nm \times Nf)/(Nm+Nf)$ (Falconer 1989). $\Delta F(1)$: rate of inbreeding using $\Delta F = 1/2 N\hat{e}$ (Falconer 1989). $N\hat{e}(2)$: Effective population sizes using $\Delta F(2) = \sum c_{i(m)}^2 / 8 + 1/(32m) - \sum c_{i(f)}^2 / 8 + 1/(32f)$ (Brown et al., 2005, Woolliams J., pers. comm.) and then $\Delta F = 1/2 N\hat{e}$ (Falconer 1989). *: $P < 0.05$, *** $P < 0.001$.

Figure legends.

Figure 1. The OVIDORPLEX system for genotyping *Sparus aurata* L. individuals with nine microsatellites. 1A: Diagram showing the design of the new multiplex PCR (OVIDORPLEX) using the Multiplex Manager software (Holleley and Geerts 2009). The base-pair space between markers is indicated by numbers. 1B and 1C: Electrophoretograms of two individuals (breeders) using the ABI3130XL Genetic Analyzer and the Genemapper 4.0 software (three artifact amplifications found in this work are show (indicated by arrows and filled): *VIC*-164bps, *NED*-167bps and *6-FAM*-199bps).

Figure 2. The *Sparus aurata* L. breeder contributions to fast- (Y axis) and to slow-growth performances (X axis) in progenies (in numbers of offspring) at 18 months of development (lines represent identical contributions to both growth groups). In circles, those breeders showing extreme observed χ^2 values compared to the statistic obtained from data sets after runs of 100,000 iterations ($P < 0.05$). (M: males, F: females).

Figures

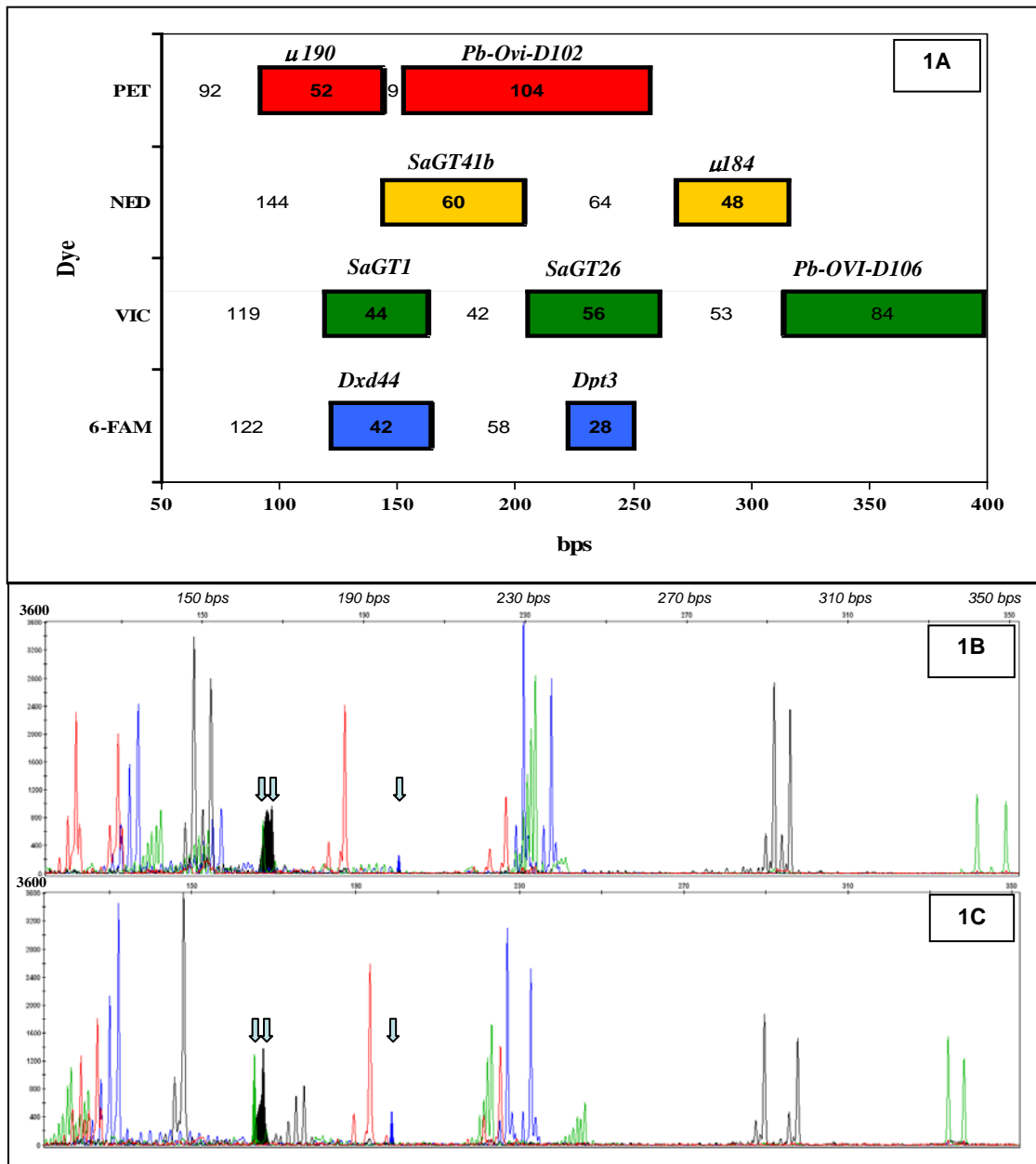


Figure 1.

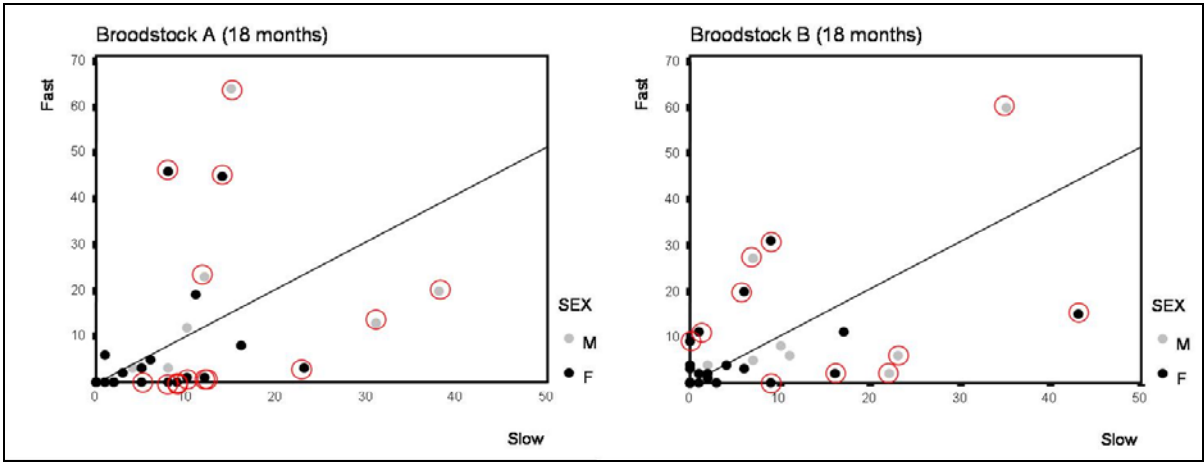


Figure 2.