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

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ABSTRACT

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

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1 Fish

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

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

The fish were moved to Universidad Politécnica de Valencia (Valencia, Spain) at the age of 165 and 157 days post-hatching, respectively, for batches A and B. They were released into 1,750-l fiberglass tanks, where they were reared under intensive conditions. The tanks were in a recirculating marine water system (30-m^3 capacity) with a rotary mechanical filter and a gravity bio-filter. All tanks were aerated, and the water temperature was maintained at 22.2 ± 2.7ºC by a heat pump that was installed in the system. The photoperiod was natural, and all tanks had similar light conditions. Fish were fed using commercial fish feed (Dibaq S.A., Segovia, Spain) by hand twice a day until apparent satiation. At an intermediate stage of growth (311 and 303 days post-hatching for batches A and B, respectively), 600 juveniles were separated by weight into four groups: A12-Fast (A12F>41g), A12-Slow (A12S<15g), B12-Fast (B12F>31g) and B12-Slow (B12S<12g). The fish were placed in four 4,000-l tanks and reared under intensive conditions to commercial size.

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

2.2 Microsatellite amplification and scoring

Genomic DNA was purified from a small piece of the caudal fin using *Chelex*^{R} 100 (Walsh et al., 1991). We developed a novel multiplex PCR (OVIDORPLEX) using the Multiplex Manager 1.0 software (Holleley and Geerts, 2009) and the gilthead sea bream microsatellite data previously collected from more than 800 gilthead sea bream individuals (Borrell et al., 2007) (Figure 1). OVIDORPLEX included nine microsatellite loci and fluorescent dyes: *VIC-SaGT1* (0.6 μM), *VIC-SaGT26* (0.3 μM), and *NED-SaGT41b* (0.5 μM) (Batargias et al., 1999); *VIC-Pb-OVI-D106* (0.6 μM), and *PET-Pb-OVI-D102* (0.4 μM) (Piñera et al., 2006); *FAM-Dxd44* (0.4 μM), and *FAM-Dpt3* (0.4 μM) (De la Herrán et al., 2005) and *NED-μ184* (0.3 μM), and *PET-μ190* (0.3 μM) microsatellites (Power et al., 2003). The QIAGEN 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* ($N_{\hat{e}}$)

The rate of inbreeding (ΔF) and the effective population size ($N_{\hat{e}}$) are related as $\Delta F = 1/2\ N_{\hat{e}}$ (Falconer, 1989). We first used the classical formula for estimating $N_{\hat{e}}$: $N_{\hat{e}} = 4$ (Nm x Nf) / (Nm + Nf) where Nm = the number of male breeders and Nf = the number of female breeders (Falconer, 1989). We also used an approach previously assessed by Brown et al. (2005) and reassessed by J. Woolliams (Roslin Institute, pers. comm.). This approach takes into account the proportion of descendants left by "presumed" unrelated parents to the next generation following Hill (1979) and Woolliams and Bjima (2000); 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 contribution of males ($c_{i\ (m)}$) and females ($c_{i\ (f)}$) to offspring and m and f the number of males and females, respectively. This formula is Woolliams´s derivation from the Hill (1979) formulation in terms of offspring contributions, and has no selection component. It simplifies to Wright's formula for Poisson family sizes and, even when it is needed to be treated with caution, it is a useful approximation to the problem of estimating $N_{\hat{e}}$ when unequal breeders contributions to offspring occur (J. Woolliams, Roslin Institute, pers. comm.; Borrell et al., 2008).

2.6 Differential parental contribution to progenies

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

3. Results

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

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

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

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

3.2 Parentage assignments and effective breeding number ($N_{\hat{e}}$) estimations

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

The effective breeding numbers, taking into account the numbers of all males and females in the tank, would be $N_{\hat{e}}$ = 31 and $N_{\hat{e}}$ = 29 in the A and B broodstocks, respectively. However, not all the breeders contributed to offspring, and this implied reductions in the $N_{\hat{e}}$ estimates (A18F: $N_{\hat{e}}\approx18$, A18S: $N_{\hat{e}}\approx26$; B18F: $N_{\hat{e}}\approx20$, and B18S: $N_{\hat{e}}\approx22$) (Table 1). We observed a globally significantly unequal contribution of breeders to progeny in both broodstocks (**A:** 19 females, 14.6 descendants expected for each female assuming equal contributions, χ^2=356.3, P<0.001; 10 males, 27.8 descendants expected for each male if there are equal contributions, χ^2=221.6, P<0.001) (**B:** 17 females, 14.0 descendants are expected for each female assuming equal contributions, χ^2=281.1, P<0.001; 9 males, 26.4 descendants expected by each male if contributions are equal, χ^2=230.9, P<0.001). A similar result was found within growth groups. Unequal reproductive success affected $N_{\hat{e}}$ estimates. The "global" effective breeding numbers calculated here using Woolliams´s approach revealed significantly lower $N_{\hat{e}}$ values than expected with equal contributions, primarily in the fast-growth groups (A18F: $N_{\hat{e}}\approx13$, A18S: $N_{\hat{e}}\approx24$; B18F: $N_{\hat{e}}\approx14$, and B18S: $N_{\hat{e}}\approx18$) (Table 1).

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

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

The individual breeder´s χ^2 values were analyzed using a re-sampling method to identify the breeders responsible for differential contribution to the fast- or slow-growth progeny groups. At 18 months of age, 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).

4.2 The breeding aspects

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

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

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

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

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

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Table 1. Growth, genetic variability parameters and effective breeding numbers (*Nê*) estimations in *S. aurata* L. after genetic analysis using nine microsatellites included in the multiplex PCR OVIDORPLEX.

Broodstock	A			B		
	P	*Fast*	*Slow*	P	*Fast*	*Slow*
Collection day (approx. months)	-	528 18	528 18	-	520 18	520 18
n	40	139	139	38	118	120
Mean Weight [Δ] (g) St. dev. St. error	1965.0 249.4 39.4	295.4 52.3 4.4	195.7 29.5 2.5	1981.5 256.6 41.6	323.8 52.7 4.8	184.1 32.4 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} *(p values)*	-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)
Sim	99.9%	-	-	99.9%	-	-
Breeders □	29♀x11♂	11♀x8♂	19♀x10♂	28♀x10♂	14♀x8♂	14♀x9♂
Nê (1)	31.2	18.5	26.2	29.4	20.3	21.9
ΔF(1) (%)	1.60	2.70	1.90	1.70	2.46	2.28
Nê (2)	-	12.7	24.6	-	14.2	17.6
ΔF(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 assignations using 95% confidence intervals. *Breeders* □: All the possible breeders among parents, breeders that left progenies after parental assignments assessments using microsatellites in progenies. *Nê(1)*: Effective population sizes using 4x(Nm x Nf)/(Nm+Nf) (Falconer 1989). *ΔF(1)*: rate of inbreeding using $\Delta F = 1/2\ N_{ê}$ (Falconer 1989). *Nê(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 $\Delta F = 1/2\ N_{ê}$ (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

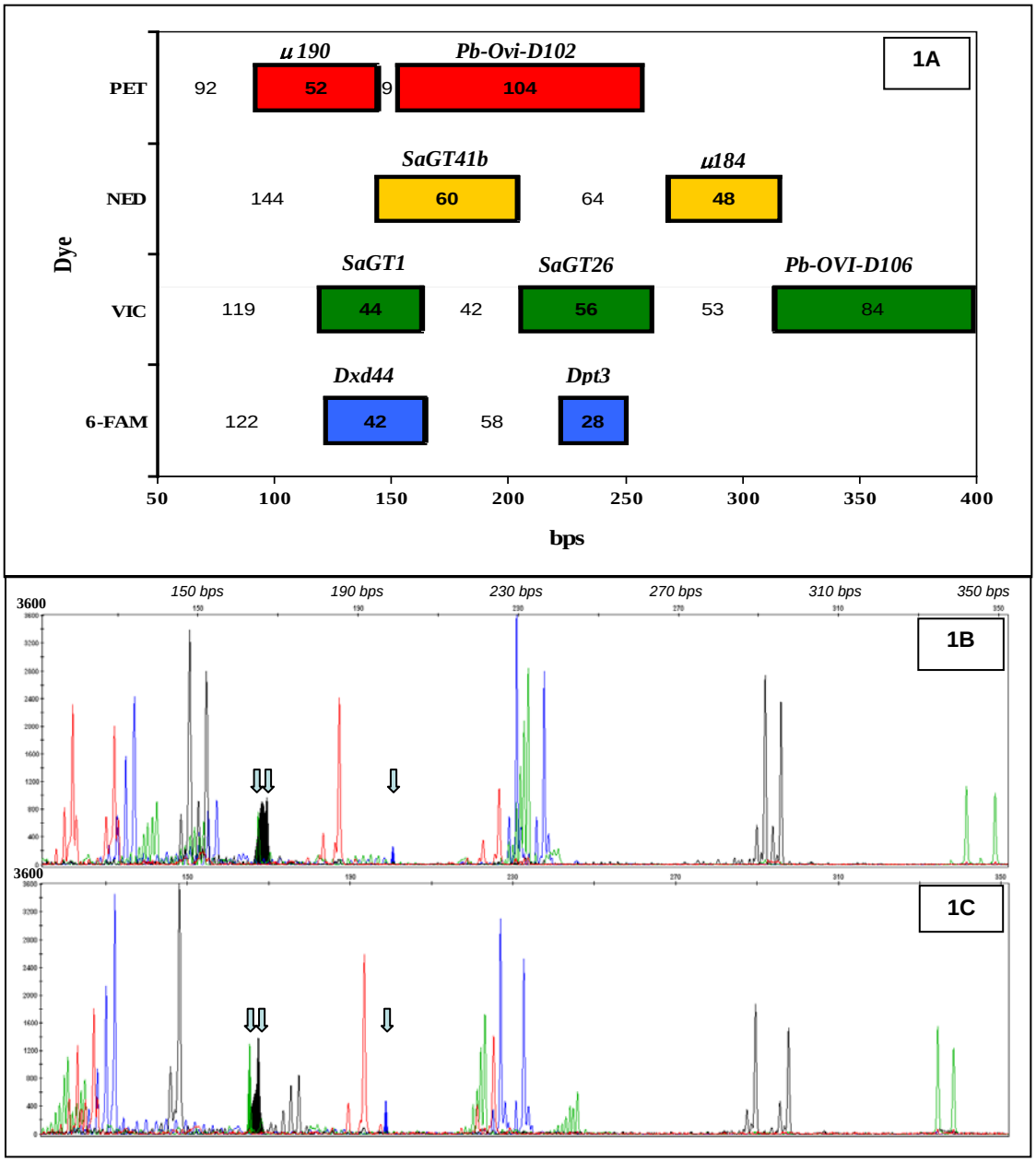

Figure 1.

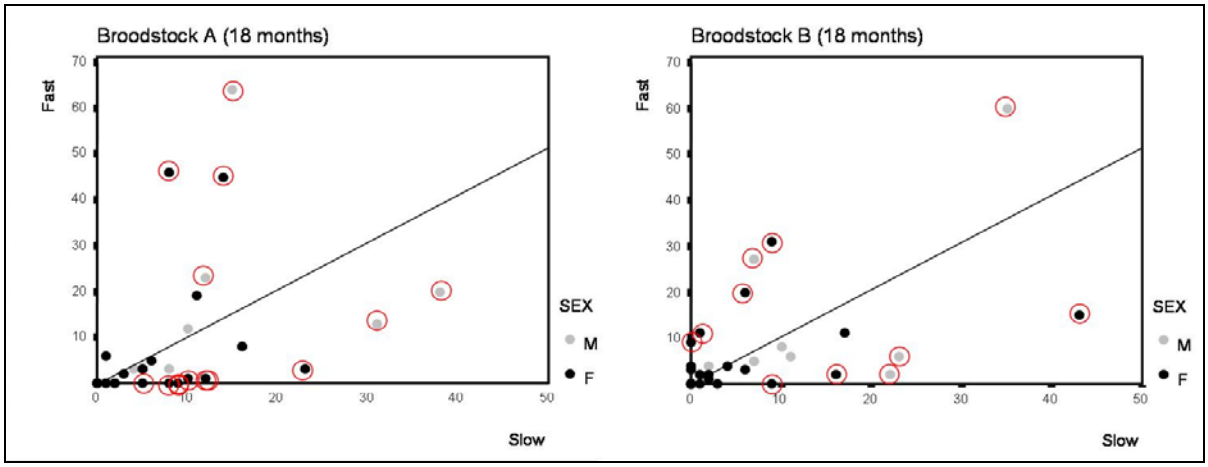
Broodstock A (18 months)
Fast
Slow
SEX
M
F
Broodstock B (18 months)
Fast
Slow
SEX
M
F

Figure 2.