Genetic Diversity among Wild and Cultured Stocks of *Sparus aurata* on Turkish Mediterranean Coasts Revealed by Mitochondrial DNA Sequences

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**Abstract**

The sequences of three mitochondrial DNA genes - 12S rRNA, cytochrome b, cytochrome oxidase II - were used to deduce genetic diversity and relationships among wild and cultured populations of gilthead sea bream (*Sparus aurata*) on Turkish coasts and coastal waters. The 415 bp of 12S rRNA, 605 bp of cytochrome oxidase II, and 557 bp of cytochrome b genes were sequenced and the data set was analyzed with Neighbor Joining and Minimum Evolution. Bootstrap analyses (1000 replicates) were performed and the relationships between samples are given in phylogenetic trees. Genetic diversity was estimated using gene diversity, number of haplotypes, and nucleotide diversity. There were no significant differences in variation in any of the three gene sequences. The maximum genetic variation occurred in cytochrome b (0.0050). Similarly, results showed very little divergence between sampling sites. Findings indicate a single wild population in the eastern Mediterranean, supporting the current classification that lacks subdivisions. Further, no differentiation between wild and culture stocks was inferred.

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Introduction

The marine gilthead sea bream (*Sparus aurata* L.) belongs to the Sparidae family which is represented by eleven genera in the Mediterranean Sea (Brown, 2003). The gilthead sea bream (*Sparus aurata*) is a subtropic species naturally distributed in the Mediterranean and Eastern Atlantic coasts of Europe and Africa (Bauchot and Hureau, 1990). It is one of the top three cultured species in Turkey and of great commercial importance. Total production in 2009 was 29,584 tons (TurkStat, 2010). Due to the commercial importance of sparids, broodstock management, genetics, and biotechnology are researched by governmental institutions, universities, and the private sector. Sea bream strains of high genetic quality have priority for fish culturists.

In Mediterranean aquaculture, the escape of cultured fish into nature occurs due to accidents or carelessness (Innocentiis et al., 2005). Also, the rearing of large individuals in sea cages may alter the genetics of wild stocks (Dimitriou et al., 2007). In Turkey, where 10 million gilthead seabream juveniles are introduced into culture annually, the potential threat to wild stocks is enhanced. Due to the relatively short history of commercial sea bream culture, phenotypic manifestations of genetic erosion may not yet be obvious. Thus, it is necessary to estimate the degree of genetic erosion in cultured stocks and its possible influence on wild strains (Alarcón et al., 2004).

Genetic structure and diversity of gilthead sea bream have been studied using microsatellites and allozymes (Alarcón et al., 2004; Innocentiis et al., 2005; Rossi et al., 2006). In the present study, we compare the DNA sequence of three mitochondrial DNA genes to estimate the level of genetic divergence and to survey the existence of subdivisions along the Turkish Mediterranean coast (Funkenstein et al., 1990).

Materials and Methods

**Fish samples and DNA extraction.** Specimens from five sampling areas along the Mediterranean coast of Turkey were collected from fish farms and the wild (Fig. 1). Locations subject to intensive commercial aquaculture including gilthead sea bream were chosen. Samples were taken to the laboratory in thermo-isolated packages with ice. Liver tissue samples were taken from each individual and stored with 95% ethanol in labeled tubes at -20°C until DNA extraction. DNA was extracted from the liver tissue using a Promega Wizard Genomic DNA Purification Kit according to the supplier’s protocol. DNA concentration and integrity were checked by NanoDrop ND-1000 spectrophotometer and 1% agarose gel electrophoresis. Following dilution to 200 ng/μl, DNA was stored at -20°C.

**DNA amplification.** Three mitochondrial gene regions were amplified by polymerase chain reaction (PCR) using pairs of primers: 12S rRNA (primer set: L1091 by 5'-AAAAAGCTTCAACTGGGATTAGATACCCCATAT-3' and H1478 by 5'-TGACTGCAGAGGTGACGGGGTGTGT-3'; Kocher et al., 1989), cytochrome oxidase II (primer set: L7450 by 5'-AAAGGAAAGGAATCGAACCCTTCC-3' and H8055 by 5'-GCTCATGAGTGGAGGACGTCTT-3'; Nomark et al., 1991), and cytochrome b (SActyB forward by 5'-TCGGAGTTGTCCTCCTCCA-3' and reverse by 5'-GTGACTGCTGGAAGGTG-3'). PCR was done in an MJ Research DNA Engine Tetrad2 thermal cycler and reactions were carried out as follows.

- For 12S rRNA: 35 cycles of a 50-μl reaction volume containing 39.6 μl sterile distilled H2O, 5 μl 10X SE PCR buffer (SibEnzyme E 332), 1 μl dNTP (10 mM; SibEnzyme N 025), 1 μl of each primer (10 pM/μl), 0.4 μl Taq DNA polymerase (5U/μl, SibEnzyme E 332), and 2 μl template. The thermal cycle profile included an initial denaturing step of
94°C for 2 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min.

- For cytochrome oxidase II (CO II): 35 cycles of a 50-μl reaction volume containing 38.85 μl sterile distilled H₂O, 3.75 μl 10X SE PCR buffer (SibEnzyme E 332), 1 μl dNTP (10 mM; SibEnzyme N 025), 1 μl of each primer (10 pM/μl), 0.4 μl Taq DNA polymerase (5U/μl, SibEnzyme E 332), and 4 μl template. The thermal cycle profile included an initial denaturing step of 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 10 min.

- For cytochrome b (Cyt b): 40 cycles of a 50-μl reaction volume containing 39.6 μl sterile distilled H₂O, 3 μl 10X SE PCR buffer (SibEnzyme E 332), 1 μl dNTP (10 mM; SibEnzyme N 025), 1 μl of each primer (10 pM/μl), 0.4 μl Taq DNA polymerase (5U/μl, SibEnzyme E 332), and 4 μl template. The thermal cycle profile included an initial denaturing step of 94°C for 5 min, denaturation at 94°C for 40 s, annealing at 50°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 5 min.

PCR products were viewed with 2% agarose gel electrophoresis to confirm the success of amplification process.

**DNA sequencing.** Double-stranded PCR products were purified by Promega Wizard SV Gel and PCR Clean-Up System (Promega A9282). Amplified products were sequenced with a Beckman Coulter CEQ 8000 Genetic Analysis System using a Beckman Coulter CEQ Dye Terminator Cycle Sequencing Kit. Primers were the same as for PCR.

**Sequence analysis.** Sequences were aligned in Sequencer 4.9 and analyzed using MEGA4. The consensus sequences were recorded in the GenBank database with accession numbers of GU902250 (12S rRNA - 415 bp), GU902251 (cyt b - 557 bp), and GU902252 (CO II - 605 bp). Phylogenetic trees were constructed with Neighbor Joining (NJ) and Minimum Evolution (ME) algorithms (Apostolidis et al., 2001). The reliability of the inferred trees was assessed by bootstrap tests with 1000 replicates (Iwi et al., 1999).

Genetic diversity among wild and cultured stocks was estimated using gene diversity, number of haplotypes, and nucleotide diversity in DnaSP v 5.0 (Rozas et al., 2003).

### Results

Each of the three gene regions of three individuals from each sampling site were sequenced for a total of 90 samples. Sequences were aligned for each gene region and alignments including haplotypes were demonstrated using online Multialin software (data available on request). The average nucleotide composition for 12S rRNA gene was A = 28.2%, T = 20.8%, G = 24.7%, and C = 26.3% with an average G+C content of 51%. For CO II, the average nucleotide composition was A = 30.2%, T = 30.4%, G = 14.5%, and C = 24.9% with an average G+C content of 39.4%. For cyt b, the average composition was A = 23.4%, T = 30.9%, G = 14.2%, and C = 31.6% with an average G+C content of 45.8%. While the number of haplotypes, gene diversity, and nucleotide diversity were similar in 12S rRNA and CO II genes, values were higher for cyt b (Table 1).

### Table 1. Genetic diversity in 12s rRNA, cytochrome oxidase II (CO II), and cytochrome b (cyt b) gene regions of cultured and wild gilthead sea bream (*Sparus aurata*) from Turkish Mediterranean coasts.

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. individuals</th>
<th>No. haplotypes</th>
<th>Gene diversity</th>
<th>Nucleotide diversity (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12s rRNA</td>
<td>30</td>
<td>5</td>
<td>0.576</td>
<td>0.00594</td>
</tr>
<tr>
<td>CO II</td>
<td>30</td>
<td>6</td>
<td>0.641</td>
<td>0.00358</td>
</tr>
<tr>
<td>Cyt b</td>
<td>30</td>
<td>10</td>
<td>0.933</td>
<td>0.00845</td>
</tr>
</tbody>
</table>

Among all sequences, 47 sites (14 in 12S rRNA, 13 in CO II, 20 in cyt b) were variable, 13 of which (1 in CO II and 12 in cyt b) were phylogenetically informative. While 12S rRNA sequences included no indels (insertions/deletions), CO II and cyt b sequences included indels totaling 150 and 34 bp, respectively. The average numbers of nucleotide differences between cultured and wild populations were 2.33 in 12S rRNA, 2.14 in CO II, and 4.63 in cyt b. Nucleotide divergence was about two-fold higher in cyt b. Likewise, genetic variation was highest in cyt b.
Two methods of analysis were used to generate molecular trees for each gene (Fig. 2); the trees inferred by both methods were congruent. Low bootstrap proportions were found by both methods, indicating no existence of subdivisions.

Fig. 2. Phylogenetic trees using two algorithms (NJ = Neighbor Joining; ME = Minimum Evolution) for mitrochronal genes (a) 12S rRNA, (b) cytochrome oxidase II, and (c) cytochrome b of gilthead sea bream (Sparus aurata) from five locations on the Turkish Mediterranean coast. Bootstrap proportions are given for each method (1000 replicates). Ad = Adana, M = Mersin, An = Antalya, B = Bodrum, I = Izmir, C = cultured, W = wild, numbers = specimen.

Discussion

Nucleotide diversity in the CO II gene was lower in cultured samples than in wild samples but the opposite was true for 12S rRNA and cyt b. Despite expected lower diversity in cultured samples, wild samples had lower rates probably because of juveniles imported from different countries to meet the demand of fish farms. While rare alleles can disappear when the same individuals are repeatedly used as broodstock (Palma et al., 2001), our findings indicate that genetic diversity in Turkish broodstocks remained high. There were no negative results of using a small group of broodstock, although the inbreeding inherent in this practice often decreases genetic variation.

Non-reported but widely known escapes from cages can affect a sampled profile of wild populations but our results show no significant genetic differences between wild and cultured populations or between the geographical locations of the sampling sites. Earlier results for Mediterranean populations were similar, using different methods (Zouros, 1999; Rossi et al., 2006; Bilgen et al., 2007).

In conclusion, divergence and phylogenetic analysis of mtDNA provided a reliable estimate of genetic diversity in gilthead sea bream from Turkish coastal areas including wild and cultured fish. While this study provides useful data for sparid genetics and taxonomy, mtDNA analyses of more genes from more specimens and more locations will expand our knowledge of the phylogenetic relationships and genetic diversity among gilthead sea bream from Mediterranean and European populations.

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References