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Parent-Offspring Relationships Following Mass Spawning of Wild Adriatic Sea Bass (*Dicentrarchus labrax*)

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Summary

We have genotyped 44 fishes caught in the wild (Novigrad Sea, Croatia) using 11 microsatellite tetranucleotide markers. They were divided in two groups and after natural mass spawning, we selected 500 offspring for genotyping following their labelling with electronic tags. All fish in the parental group could be identified based on unique genotypes, and a significant number of private alleles, the alleles found only in one fish, greatly facilitated subsequent parent-offspring assignments. The majority of potential parents left no progeny, while just six of them generated over 70 %. Such highly skewed reproduction success, observed also in other studies, can quickly lead to a highly inbred population in just a few selection cycles. This kind of genetic analysis is helpful for planning of future selective breeding, where faster progress will be made possible based on the soon to be completed sea bass genome sequence.

Key words: sea bass, selective breeding, genotyping, microsatellites

Introduction

European sea bass (*Dicentrarchus labrax*) inhabits coastal waters of the southeastern Atlantic Ocean and Mediterranean Sea. In Croatia, initial life cycle studies for farming of the sea bass started in the 1960s at the Institute of Fish Technology in Zadar. Today Croatian aquaculture produces annually about 2600 tonnes of this fish, while Greece, Turkey and Italy are the leading producers worldwide. The total production of sea bass has reached about 100 000 tonnes, corresponding to the sales value of about €320 million (*1*). In the Mediterranean countries, sea bass and gilthead sea bream (*Sparus aurata*) represent the most valuable fish species for aquaculture.

Reliable, sustainable and profitable mass production of sea bass depends on proper control of many parameters; one of them is genetic quality of the broodstock. The reproduction process typically relies on natural mating and mass spawning in a tank containing several dozen of dams and sires selected and maintained specifically for that purpose. While in early days of sea bass aquaculture the selection of dams and sires was based mainly on the growth rate, body mass and shape, today this selection frequently includes the results of genetic analyses.

The genome of sea bass contains about 600 million base pairs (*2*), and the efforts to complete its sequence and annotation are currently in progress using several approaches. Thus, next generation sequencing (NGS) technology (Illumina Solexa, San Diego, CA, USA) was used to generate a 20 times genome coverage of a sea bass specimen belonging to the Adriatic clade (*3*). Sanger end sequencing of a sea bass genomic BAC-library allowed comparative mapping using as a reference the well-annotated stickleback genome (*4*). Radiation hybrid mapping based on over 1500 markers provided a high resolution physical map of the sea bass genome (*5*). Three of the sea bass chromosomes have been directly sequenced

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and annotated using a combination of Sanger sequencing and Roche/454 pyrosequencing (*4*). Rapid advances in NGS technologies, in particular single molecule real time sequencing (Pacific Biosciences, Menlo Park, CA, USA), will facilitate the completion of the sea bass genome sequencing project in the near future. NGS will continue to have a strong impact not only on the studies of fish genomes, but also of fish transcriptomes (*6*). Over 30 000 expressed sequence tags (ESTs) derived from 14 tissues of sea bass have been described recently (*7*).

In the massive amount of generated DNA sequence data, there are thousands of potential markers that can link desirable phenotypic traits to the genotype. Establishing such links will remain a major challenge for researchers in the years to come. In sea bass, the heritability of growth trait is high, varying from 0.29 to 0.60 (*8,9*), and a proper selection may achieve doubling of the growth rate in just four generations (*1*). Usually fish body mass is taken to estimate the growth trait, with the underlying assumption that in a group of fish of the same age, those fishes which are heavier also grow faster. Significant differences in body size are observable in the early growth phase among fishes of the same age, and it is a common practice in sea bass aquaculture to perform size selection at multiple times. It has been found that in this fast-growing phase of juvenile sea bass, individuals of different starting body mass grow at equal rates (*10*). This makes questionable the practical application of the growth rates calculated in a classical way, but Bavčević *et al*. (*11*) found that the use of von Bertalanffy coefficient overcomes this problem, making possibile the comparison of growth rates among the fishes having different starting lengths.

Using a panel of 31 microsatellite markers, Massault *et al*. (*1*) mapped two quantitative trait loci (QTL) for body mass to linkage groups (chromosomes) 4 and 6. Two other traits, morphometric and stress, were mapped as well (*1*). The availability of single nucleotide polymorphism markers (SNPs) will allow more precise mapping due to their high density; thus over 20 000 SNPs were identified in three sea bass chromosomes and ESTs (*3, 12*).

It is important to keep in mind that some desirable traits might be related to gene expression rather than to gene sequence. For example, the switch from fish oil to vegetable oil in salmon diet caused changes in expression level of a number of genes in the liver (*13*), and so did the switch from fish proteins to plant proteins in rainbow trout feed (*14*). The replacement of fish with vegetable feed is desirable for further increase of sustainable fish farming in general (*15*). Another interesting example relates to the well-known fact that fish sex ratio shifts in response to temperature, so that a higher number of males is produced at higher temperatures. Recently, the molecular basis of this observation was elucidated in European sea bass: the promoter of the gonadal aromatase (*cyp19a*) gene is methylated at a high temperature, leading to reduced expression of the gene coding for the enzyme that converts androgens to estrogens (*16*).

Given the high heritability of some important traits in sea bass, it is essential to identify parent-offspring relationships, especially in broodstock selection. Controlled mating of single fish pairs followed by separate rearing of the offspring under identical conditions poses significant challenges in terms of technology and cost. Therefore, it is preferable to identify parents of selected offspring after mass spawning and common rearing, at the stage when the offspring reach a size suitable for labelling with electronic tags. For parentage testing in general, during the past 20 years microsatellites have been used as the markers of choice. They represent DNA sequences containing many repeats of one identical motif that is 2–6 bases long. Individuals differ in the numbers of the repeats, and these numbers correspond to those possessed by their parents. Microsatellite markers are widely employed in selective breeding (*17,18*) and studies of populations (*19,20*). Here we describe genotyping results of a population of 44 fishes caught in the wild together with the identification of some of their offspring using 11 microsatellite markers.

Material and Methods

Sea bass growth

The sea bass specimen (44) caught in the wild (Novigrad Sea, Croatia) were tagged and a fin sample was taken for genotyping. The fishes were divided into two groups prior to the spawning season and kept in tanks having a volume of about 10 m^3. Spawning was successful in one tank containing 17 dams and 6 sires. The larvae were first reared in three tanks until day 30, and then transferred into two tanks until day 174 post fertilization, when they were moved to a farming site at the sea. After the fish reached an average length of 14.5 cm, about 500 fishes that were longer than the average were tagged for genotyping, their total length measured, and the fish transferred to a new cage. Feeding was performed automatically for 10 h each day. After 168 days, fish total length was measured again, and a fin sample was taken for genomic DNA isolation.

Genotyping

Genomic DNA was isolated according to common protocols. For genotyping we first screened 15 tetranucleotide microsatellite loci whose sequences were described (*21;* GenBank, NCBI, Bethesda, MD, USA), and then chose 11 of them that were the most polymorphic. The primers were redesigned to give amplified products in the size range from about 100 to 220 bp, so that the electrophoretic separation could be carried out on short, native, high-resolution polyacrylamide-based gels that also offer accurate sizing of the alleles (*22*). The gels were made at Gentius d.o.o., Zadar, Croatia. After PCR amplification, the samples were mixed with loading buffer (50 % sucrose in 20 mM Tris-acetate-EDTA (TAE) buffer) and typically 50 samples were loaded onto one gel. The gels were typically run in 60 mM TAE buffer at 200 V for 80 min, stained with SYBR Gold (Invitrogen™, Life Technologies, Carlsbad, CA, USA), photographed and analyzed using TotalLab gel analysis software (TotalLab Ltd, Newcastle upon Tyne, UK). The allele data were analyzed with GeneAlEx (*23,24*), and parentage assigned with the help of WHICHPARENTS software (Bodega Marine Laboratory (BML), UC Davies, Bodega Bay, CA, USA).

Results

For genotyping we performed PCR at individual microsatellite loci and separated the amplification products on wide but short high-resolution gels (Fig. 1). The absolute sizing accuracy typically achieved was ±1 bp of the fragment length expected from its DNA sequence, allowing reliable allele calling.

In this fish population, the number of alleles found varied from four at the AY529495 locus to 17 at the AY-639106 locus, as shown in Table 1. The observed heterozygosity was moderate to high at most loci. Significant deviations from Hardy-Weinberg equilibrium were noted at two loci, AY387400 and AY639106. There were 17 private alleles in this sea bass population, and each fish could be identified based on its unique genotype. The combined probability of identity at the 11 loci was $6 \cdot 10^{-11}$.

The identification of parent-offspring relationship in this fish population was greatly facilitated by the presence of a large number of private alleles among the parents. Data analysis showed that of the 44 potential parents, just six of them produced over 70 % of the offspring. Moreover, dams 6 and 39 with sires 12 and 14 produced over 50 % of all offspring, as can be seen from Fig. 2. Evidently, the contribution of parents to the offspring population is greatly unbalanced, and the majority of potential parents left no offspring.

Discussion

Prior to genotyping, the offspring were selected based on their above average length and then electronically tagged. It is thus possible that the observed skewed parental contribution was the result mainly of that selection. On the other hand, it is also possible that in the whole offspring population the contribution of just a few parents greatly dominated over all the others. This second possibility seems more likely in view of the results of other studies. Thus, after natural mating of 57 sea bass parents followed by genotyping of the progeny, Massault *et al.* (*1*) identified five males and two females giving rise to eleven large full-sib families. Even more drastic results were reported by Chatziplis *et al.* (*25*): one

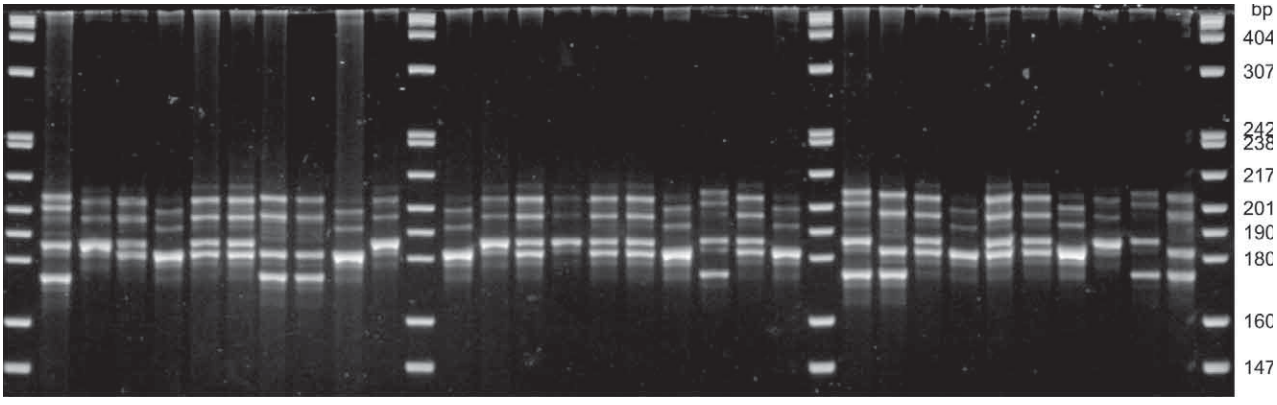

Fig. 1. An example of electrophoretic separation used for genotyping of 30 sea bass offspring at the AY636156 locus. The marker is pBR322/MspI digest, with the fragment lengths indicated in base pairs. The less intense bands above the allele bands represent heteroduplexes; weak heteroduplex bands are visible also in homozygous samples due to small amounts of stutter bands noticeable below the allele bands

Table 1. The loci, their sequences and numbers of repeats in the sequences were taken from the data available at GenBank

Locus	Repeat motif	Number of repeats	Number of found alleles	The most common allele	Ho	He
AY639098	CAGA	9	9	12 (0.273)	0.886	0.830
AY639103	TTTC	15	8	15 (0.205)	0.909	0.820
AY694149	GAAA	15	7	15 (0.693)	0.545	0.493
AY714331	TTGG	8	5	5 (0.705)	0.523	0.478
AY387400	CAGA	9	9	15 (0.409)	0.682	0.764
AY529495	GACA	7	4	7 (0.500)	0.659	0.619
AY262085	CTGT	10	7	10 (0.750)	0.500	0.419
AY639099	GTCT	20	13	10 (0.648)	0.568	0.559
AY636156	CTTT	12	6	11 (0.443)	0.773	0.708
AY453619	TGTC	8	8	14 (0.443)	0.727	0.742
AY639106	TCTA	20	17	17 (0.102)	0.977	0.910

The percentage of the most common allele, relative to all found alleles at that locus, is given in parentheses. Ho=observed heterozygosity, He=expected heterozygosity

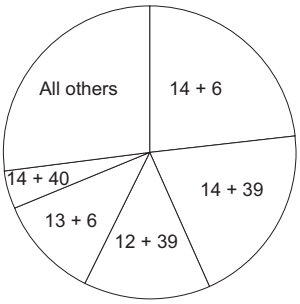

Fig. 2. The contribution of particular parents to the offspring population. The numbers refer to fish individuals. In addition to the 6 major contributors, 5 other parents were identified. The second parent of those offspring (offspring under 'All others') was in almost all cases one of the most productive 6 parents shown above

dam contributed to nearly 95 % and one sire to about 50 % of the genotyped progeny, from the broodstock consisting of 58 dams and 45 sires. Similar skewed parental contribution after mass spawning has been reported for other fish species, for example sole (*26*), Atlantic cod (*27*), salmon (*28*) and gilthead sea bream (*29*).

The selective breeding program for sea bass usually aims to maximize the mass and length at harvest, and these traits have been reported to have a moderate to high heritability (*1,8,9,25,30,31*). In view of the finding that final length and growth rate are not necessarily proportional (*10,11*), it is important to properly design the monitoring of a selected trait so that stochastic processes at early larval stages do not obscure or compromise the impact of heritability. On the other hand, given the above-described unbalanced parental contribution to the offspring population, there is a serious risk of the loss of genetic diversity after just a few generations. The risk of inbreeding cannot be avoided by partial replacement of broodstock with fish from the same farmed population. But a replacement with wild fish may lead to a loss of the gain achieved through the selection program. Evidently, it is necessary to find a right balance between the maintenance of genetic diversity and the selection of the fish that possess desired traits. Some of these traits may be expressed later in life, like meat texture, feed efficiency and resistance to diseases. These traits might be diminished or compromised if the selection for those traits that are expressed early in life becomes associated with high inbreeding.

The identification of full-sib and half-sib families is the essential requirement for performing marker-assisted search for desired quantitative trait loci, as practiced in several such studies involving sea bass (*1,8,9,25,30,31*). Recently, following the trend common in other fish species, two reports appeared on genotype-diet interactions in sea bass fed with vegetable feed. One study reported a low genotype-diet interaction, as those fishes which gained mass faster on a diet containing marine products also gained it faster when given only a plant-based feed (*32*). In contrast, another study reported that two half-sib families that showed similar growth rate on a fish-based diet displayed significantly different growth rates when fed a plant-based diet (*33*). Additional studies are evidently needed to address this important question.

Genetic markers are useful not only for parentage assignment, population studies and finding quantitative trait loci, but also for fish tracking. With sequencing of the sea bass genome nearing completion, enormous number of polymorphic markers will soon become available for identification of individual fish from a population. Even with a limited number of microsatellite markers described here, we have been able to tentatively assign fishes to several populations thanks to the presence of private alleles in different sea bass populations (unpublished results). It is likely that with a suitable choice of additional genetic markers, like SNPs and/or indels, in the future it will be possible to trace each individual fish to the farm at which it was produced.

Conclusion

This work illustrates that the use of genetic markers can answer some important questions that are unanswerable by other methods. The importance is related not only to commercial benefits for fish producers, but also to the benefits for consumers of farmed fish. Our better understanding of the farmed and wild fish populations is a necessary condition for long-term sustainable exploitation of this sea resource.

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