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in

Bartley D.M. (ed.), Basurco B. (ed.).
Genetics and breeding of Mediterranean aquaculture species

Zaragoza : CIHEAM
Cahiers Options Méditerranéennes; n. 34

1998
pages 153-168

Application of molecular markers to aquaculture and broodstock management with special emphasis on microsatellite DNA

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SUMMARY - The use of molecular genetic markers to address questions related to aquaculture management has steadily grown over the last two decades. These markers can provide valuable information for various aspects of aquaculture practice, such as: (i) genetic identification and discrimination of aquaculture stocks; (ii) monitoring of inbreeding or other changes in the genetic composition of the stocks that may result from such phenomena as breeding programmes, founder events and genetic drift; (iii) comparisons between hatchery and wild stocks; (iv) assessment of the impact on natural populations of escaped or released cultured fish; (v) assignment of progeny to parents through genetic tags, so that animals from different families can be reared together in breeding programmes; (vi) identification of marker genetic loci associated with quantitative trait loci (QTL) and use of these markers in selection programmes (marker assisted selection); and (vii) assessment of successful implementation of genetic manipulations such as induction of polyploidy and gynogenesis. Allozymes were the first molecular genetic tools used in aquaculture management. Mitochondrial DNA (mtDNA) provided another valuable marker, because of increased resolution and discrimination power in studies of population structure. Nuclear DNA markers were more recently introduced in aquaculture studies and their main attractiveness lay in their abundance in the genome, their Mendelian inheritance, and their potential to detect high levels of polymorphism. Especially microsatellite DNA analysis revolutionized the use of molecular genetic markers in aquaculture and seems to be the marker destined to dominate this type of studies in the coming years. The application of genetic markers to aquaculture management is demonstrated here by the analysis of genetic variability in cultivated and wild populations of gilthead sea bream (*Sparus aurata*).

Key words: Molecular genetic markers, aquaculture management.

RESUME - *"Application de marqueurs moléculaires en aquaculture et en gestion des reproducteurs". L'utilisation de marqueurs génétiques moléculaires pour traiter les questions liées à la gestion aquacole a trouvé une application qui s'élargit régulièrement pendant ces deux dernières décennies. Ces marqueurs peuvent apporter une information précieuse concernant plusieurs aspects de la pratique aquacole, tels que : (i) identification génétique et discrimination des stocks aquacoles ; (ii) suivi de la consanguinité ou d'autres changements dans la constitution génétique des stocks, résultant de phénomènes tels qu'événements concernant le fondateur ou dérive génétique ; (iii) comparaison entre stocks d'écloseries et stocks sauvages ; (iv) évaluation de l'impact de poissons d'élevage échappés ou lâchés sur les populations naturelles ; (v) identification de l'apparentement, en assignant une étiquette génétique héritable à chaque poisson individuel, le résultat étant que des animaux de familles différentes peuvent être élevés ensemble dans le cadre de programmes de sélection ; (vi) identification de loci comme marqueurs génétiques, associés à des loci de caractères quantitatifs (QTL) et utilisation de ces marqueurs dans le cadre de programmes de sélection (sélection assistée par marqueurs) ; and (vii) évaluation de mises en oeuvre réussies de manipulations génétiques telles que l'induction de polyploïdie et de gynogénèse. L'analyse d'allozymes a été le premier type d'outil génétique à être largement utilisé pour la gestion en aquaculture. L'ADN mitochondrial (mtDNA) a constitué un marqueur de choix pendant de nombreuses années, car il présente plusieurs caractéristiques qui augmentent le pouvoir de résolution et de discrimination dans des études de structure de population. Les marqueurs nucléaires d'ADN ont été introduits bien plus récemment dans les études aquacoles et leur principal attrait réside dans leur abondance dans le génome, leur hérédité mendélienne, et leur potentiel pour détecter de hauts niveaux*

*de polymorphisme. Les analyses à l'aide d'ADN microsatellite en particulier ont marqué une révolution dans l'utilisation de marqueurs génétiques moléculaires en aquaculture et semblent représenter le type de marqueur destiné à prévaloir dans ces études pendant les prochaines années. L'application de marqueurs génétiques à la gestion aquacole est illustrée par l'analyse de la variabilité génétique chez des populations élevées et sauvages de daurade (*Sparus aurata*).*

Mots-clés : *Marqueurs génétiques moléculaires, gestion aquacole.*

Introduction

Looking thirty years back, one realizes that the word "molecular" in the titles of the 1966 seminal papers by Hubby and Lewontin (1966) and Lewontin and Hubby (1966) was both misleading and visionary. Misleading, because no molecular biologist would ever consider scoring allozyme variation as part of his/her trade. Visionary, because today's population biologists can collect their primary information at the most basic and decisively molecular level: the nucleotide. It is possible that in the future we will be able to isolate and characterize directly genes responsible for phenotypic characters, such as growth capacity, disease resistance, etc. But many topics in organismal biology can already be addressed effectively by examining variation in "randomly chosen" DNA's or proteins, in other words by employing "molecular genetic markers" (Avise, 1994). Scientists applying this approach today have a rather wide and diverse collection of tools at their disposal. But as this collection grows, the choice of tools becomes more difficult. It is not always the case that the appearance of a new method renders another method obsolete. Indeed, one may argue that the possibility of simultaneous use of two or more assays of scoring genetic variation may shed light on questions that neither assay could answer alone.

The use of molecular genetic markers to address questions related to aquaculture management has found a steadily widening application in the last two decades. These markers can provide valuable information for various aspects of aquaculture practice, such as genetic identification and discrimination of aquaculture stocks, monitoring the consequences of founding and propagation of aquacultured stocks, assisting selective breeding programmes, and assessing chromosomal and gene manipulations such as induction of polyploidy and gynogenesis (Ferguson, 1994). Another important application of genetic markers in aquaculture is the assessment of the impact on natural populations of escaped or released cultured fish.

The purpose of this communication is to provide a comparative evaluation of the various techniques now widely used in population genetics in general and in aquaculture practice for effective management of stocks in particular. Salmonids have been the subject of most of the work undertaken world-wide in this research area . Here we will first list the various types of markers together with what we consider to be their advantages and disadvantages and then present briefly the main aspects of aquaculture practice, where these markers can be applied, giving also examples from our own work on a Mediterranean cultured species, the gilthead sea bream (*Sparus aurata*). It is not our intent to review thoroughly the use of molecular genetic markers in aquaculture. For recent excellent reviews on this topic we refer readers to Wright (1993), Carvalho and Hauser (1994), Ferguson (1994), O'Reilly and Wright (1995).

Tools

Effective genetic management of cultured fish often involves the analyses of distinct alleles at defined loci (Ferguson, 1994). In the following paragraphs we will present in general lines the most important of the wide variety of laboratory assays used towards this end. Each assay corresponds to a specific type of genetic marker.

Allozymes

Allozymes are electrophoretically distinguishable protein variants. The advent of protein electrophoresis in the late 1960's resulted in the first application of genetic markers to the management of reared fish. As a result, the approach to difficult discriminatory problems was shifted from morphology to direct products of the gene (Wirgin and Waldam, 1994). The simplicity and general applicability of the technique have made this the most widely studied form of molecular variation. Any source of soluble proteins, from bacterial cultures to animal fluids, is in principle suitable for allozyme analysis and the protocols of electrophoretic separation and staining are easily adjustable from species to species. The genetic interpretation of allozyme profiles (zymograms) is also straightforward. One major drawback has been the inability to read genotypes from small quantities of tissue, which makes allozymes inapplicable for small organisms or for the immature stages (e.g., larvae). But the main disadvantage, one that appears to be intrinsically difficult to overcome, is that only a small fraction of enzyme loci appear to be allozymically polymorphic in many species.

Murphy *et al.* (1990) list 75 enzyme systems, coded by several hundred genetic loci, that may potentially be analyzed in fishes. It may soon become possible to record amino acid polymorphism in large scale surveys by direct sequencing rather than by electrophoretic separation of the peptide product. This will increase enormously the number of protein loci that could be surveyed. Because allozymes cannot be assumed to be selectively neutral (e.g., Karl and Avise, 1992, and references therein) and because the amount of their polymorphism is limited (these two aspect are obviously related), they are not the assay of choice for the study of the biogeography of wild populations or the discrimination of aquacultured stocks. Nevertheless, allozyme analysis continues to be the method of choice for several applications in fisheries and aquaculture (Utter, 1991).

Mitochondrial DNA (mtDNA)

By the early 1980's examination of the gene itself became possible by determining directly or indirectly differences in the nucleotide sequence of DNA molecule. One of the findings that arose from early studies was that the DNA of mitochondria (mtDNA) is characterized by high levels of sequence diversity at the species or infra-specific levels, despite great conservation of gene function and arrangement (Wirgin and Waldam, 1994, and references therein). Mitochondrial DNA became a very popular marker and dominated genetic studies designed to answer questions of phylogeny and population structure in fish for more than a decade. Three properties of mtDNA set it apart from nuclear DNA: it occurs in

multiple copies in each cell (in contrast to two copies for a "single copy" nuclear locus), it is transmitted uniparentally, and it does not recombine. Moreover, it evolves much faster than coding regions of DNA (for more details see Brown *et al.*, 1982, Attardi, 1985, Moritz *et al.*, 1987, Avise 1994). Presence of multiple copies does not, however, translate into a large variety of copies within the cell. For reasons not fully understood, the speed with which the maternal lineage of a heteroplasmically conceived individual becomes homoplasmic is rather high. As a result, we can speak of the "mitotype" of an individual in the same way as we speak of its (nuclear) genotype. One consequence of uniparental transmission is that the effective population size for mtDNA is smaller than that of nuclear DNA (Moritz *et al.*, 1987), so that mtDNA variation is a more sensitive indicator of population phenomena such as bottlenecks and hybridizations. Sex-specific differences in gene flow could also be revealed by contrasting nuclear with mitochondrial DNA. In a species in which mtDNA is maternally transmitted but gene flow occurs mainly or exclusively through males, divergence among populations is expected to be much higher for mtDNA than nuclear DNA. This, at the same time, means that a disadvantage of this marker is its inability to detect male mediated genetic mixing of stocks.

Nuclear DNA (nDNA)

Nuclear DNA markers were more recently introduced in aquaculture studies and their main attractiveness lay in their abundance in the genome (theoretically DNA sequences offer on average more than 3 billion characters suitable for scoring variation, Wirgin and Waldam, 1994), their Mendelian inheritance, and their potential to detect high levels of polymorphism. The majority of nDNA markers comprise regions of the nuclear genome not encoding for a protein product, and, therefore, assumed to be under relaxed selective constrains. This allows these sequences to evolve more rapidly than the coding sequences and therefore renders them suitable for assessment of genetic structuring at the species or lower level. The most commonly nDNA markers used for aquaculture applications are listed below.

Tandemly repeated DNA (mini- and micro-satellites)

Tandemly repeated blocks of DNA of identical or similar sequence are dispersed throughout the genome of most, if not all, eukaryotic organisms (O'Reilly and Wright, 1995, and references therein). Three different classes of this repetitive and highly polymorphic DNA have been distinguished traditionally, based on the size of the repeat unit: a) satellite DNA, which refers to long repeat units (hundreds or thousands of base pairs), b) minisatellite DNA or variable number of tandem repeat (VNTR) DNA (Jeffreys *et al.*, 1985; Nakamura *et al.*, 1987), which refers to genetic loci with repeats of smaller length (10-64 bp), and c) microsatellite DNA or simple sequence or short tandem repeat (STR) DNA (Litt and Luty, 1989; Tautz, 1989; Edwards *et al.* 1991), in which the repeat unit is only 2 to 4 bp long. The term VNTR is frequently used for both mini- and microsatellite DNA. Length variation in tandemly repeated DNA, particularly in the case of mini- and microsatellites is usually due to the changes (increases or decreases) in the copy number of repeat unit (Jeffreys *et al.*, 1988)

The term "DNA fingerprinting" was originally associated with the approach of Jeffreys *et al.* (1985), in which Southern blot/hybridization assays of minisatellite regions of DNA (after restriction digestion of individual genomic DNA) reveal multi-locus gel banding profiles that distinguish most or all individuals within a sexually reproducing species (Avise, 1994). The original Jeffrey's probes, which hybridized to conserved core sequences, 10-15 bp long, were isolated from a myoglobin intron in humans, but it was found that they also cross-hybridized in many other species, fishes included (e.g., Baker *et al.*, 1992). Subsequently there has been an explosion in the development of new methods for the production of species- and individual specific DNA fingerprints. However, profiles resulting from multi-locus DNA fingerprinting are very complex and this puts severe constrains in the analysis and interpretation of the results. It is almost impossible to identify both members of allelic pairs at individual loci and therefore estimation of allelic frequencies are not obtained. These limitations render multi-locus fingerprinting rather unsuited for population level applications (Wright, 1993). Moreover, quite often the results of multi-locus fingerprinting protocols are not reproducible. As a result, the development of single-locus profiling techniques was sought, in which allelic variation is surveyed at individual VNTR loci. Two approaches have been used: a) Southern blotting and hybridization using as a probe DNA from a single VNTR locus, preferably the unique flanking regions, and b) by PCR amplification of the locus using primers flanking the repeat array and separation of the PCR products by gel electrophoresis (O'Reilly and Wright, 1995). Single-locus approaches obviate most of the problems associated with multi-locus methods; nevertheless some limitations still exist, mainly because often alleles do not differ from one another by discrete, integral increases or decreases in the number of repeat copies (Jeffreys *et al.*, 1988). This makes the comparison of alleles sizes between gel difficult, and has necessitated the binning of alleles into defined size classes (O'Reilly and Wright, 1995, and references therein).

The first to demonstrate polymorphism at microsatellite loci were Tautz (1989) and Weber and May (1989). The use of this class of markers was explosively expanded, mainly due to their ease of scoring through PCR, their high polymorphism and the accuracy of allele characterization. Microsatellites differ from minisatellites inasmuch as the repeat unit is very simple (mostly two, but also three or more nucleotides), and the total length of the "locus" is much smaller than in minisatellites. Most importantly, microsatellites are much more numerous in the genome (particularly of vertebrates). In general microsatellites are justifiably considered as the ideal markers for a wide range of applications in fisheries and aquaculture. They are optimal for mapping "causal" genes, whether these are responsible for single factor conditions (e.g., muscular dystrophy in humans) or for multifactorial traits (quantitative trait loci, QTL). Once loci closely linked to QTL have been found they can be used for marker-assisted-selection programmes (Cheverud and Routman, 1993). They are also the best markers for determining parenthood in mass-crosses (Batargias and Zouros, 1993, Batargias *et al.*, 1997), tracing escapes form contained to wild populations and estimating coefficients of kinship among individuals drawn from a population (Blouin *et al.*, 1996). Their basic drawback remains the high cost and labor-intensiveness of the first phase of the technique, i.e. the development of primers. This is to some extent counter-balanced by the usually good crossability of primers in related species. Another disadvantage of microsatellites is the existence of null alleles, that is alleles that do not amplify in

PCR reactions. In some cases the presence of a null allele can be detected as heterozygote deficiency, and then new primers can be designed to obviate this problem. But quite often the existence of null alleles go unnoticed, whereas heterozygote deficiency may be caused by other factors, such as population substructuring (O'Reilly and Wright, 1995).

Anonymous nuclear DNA markers.

Under this category we include assays that target a segment of DNA of unknown function. The segment can be amplified from individual specimens and the polymorphism scored as length difference of the PCR products. Alternatively, the product may be digested by a set of restriction enzymes and the polymorphism scored as restriction fragment length polymorphism (RFLP). The primers are usually designed from sequences originally obtained for other purposes. Another method is to use the PCR product from a reference individual as a probe against digested total DNA from the sampled individuals. This calls for a more cumbersome protocol, but has the potential to uncover more polymorphism. cDNA probes represent a special version of this technique. Individual clones from a poly-A messenger RNA cDNA library are amplified by using vector primers and used as probes against Southern transfers (for examples see Pogson and Zouros, 1994, Pogson *et al.*, 1995).

The method of randomly amplified polymorphic DNA (RAPD, Welsh and McClelland, 1990) uses short primers of arbitrary sequence to amplify anonymous regions of genomic DNA. It is a fast and cheap assay, but the penalty for this convenience is poor reproducibility and ambiguity in the interpretation of results. The profiles are usually multibanded and polymorphism is scored as presence/absence of specific bands. As a result, most population genetics models cannot be applied, and analysis is based on phenotype rather than allelic frequencies. RAPDs are more suitable for species and subspecies comparisons, than for intra-species population studies.

Applications

Genetic discrimination of stocks

A fundamental requirement in any long term culture programme is the genetic identification and discrimination of stocks. If the deleterious effects of inbreeding are to be avoided, crossing fish from genetically different strains is of critical importance. This can be done most effectively if knowledge about genetic similarity or difference between the strains is available, especially when pedigree information is lacking (Ferguson, 1994). At the same time, genetic markers can be suitable for assessing the differences between aquacultured stocks and wild populations and addressing concerns about escapes or releases from aquaculture farms into natural populations. Another possible application of the genetic analysis of cultured stocks, by means of molecular markers, is the development of genetic tags, with which breeders could safeguard their propriety rights of their products.

Allozyme markers have been used extensively for studies in salmonids. Some systems allowed discrimination between European and North American *Salmo salar,*

and northern and southern European groups have been also identified, while in certain cases evidence has been obtained that these sytems can distinguish populations at the local level (Wilson *et al.*, 1995 and references therein). However, it has been demonstrated that allozymes have lower discriminatory power than mtDNA markers, because polymorphism is lower and stocks are usually not fixed for different alleles. The generality of this observation is not easy to be assessed (Ferguson, 1994, and references therein). Regarding Mediterranean species, Reina *et al.* (1994) used allozyme markers to assess the phylogenetic relationship of gilthead sea bream (*Sparus aurata)* with other members of the Sparidae family. Previously, Basaglia and Marchetti (1991) and Basaglia (1992) have used, for the same purpose, electrophoretic profiles of soluble non-specific proteins. Phylogenetic relationships of sparid species were also investigated by Garrido-Ramos *et al.* (1995), using as a marker two centromeric satellite DNA families.

Mitochondrial DNA has found extensive use for studying population structure of fishes (for a review see Meyer, 1993). Most of the work for aquacultured species was done with salmonids (Ferguson, 1994, and references therein). Concerning *Sparus aurata,* Funkenstein *et al.* (1990) have reported the first mtDNA polymorphism in an Israeli broodstock, by using RFLP analysis of the whole mtDNA molecule. Later, Magoulas *et al.* (1995) confirmed this polymorphism in a Greek broodstock and proceeded to produce the first restriction map of the mtDNA molecule of this species. In addition, these authors provided the nucleotide sequence of a 350 bp segment from the cytochrome b gene. By combining restriction enzyme analysis of the whole mtDNA molecule with direct sequencing of a region of Cyto b gene, after PCR amplification, eight mitotypes were detected between the breeders (Table 1). Statistical analysis revealed no significant difference between the frequencies of the restriction mitotypes in Israeli and Greek stocks, and this, coupled with the fact that both broodstocks originated from local wild populations, suggests that the Eastern Mediterranean populations of this species may not be highly differentiated.

Table 1. Joint mitotypes of *Sparus aurata* broodstock from Greece, resulted from cytochrome b sequence (capital letter) and the restriction profiles (lower-case letters) (modified from Magoulas *et al.*, 1995)

Joint mitotype	Number of animals
Aaaaaa	15
Abbaaa	1
Baaaaa	1
Bbbaaa	6
Caaaaa	1
Daaaaa	1
Eaaaaa	4
Ebbaaa	1

Patarnello *et al.* (1993) analyzed, by direct sequencing, variability in the Cyto b gene of the second most important marine fish cultivated in the Mediterranean, the European sea bass (*Dicentrarchus labrax*). They examined individuals sampled from both wild and farmed populations and found evidence for differential mitotype distribution between natural and farmed stocks.

The application of *mini-* and *microsatellite DNA* markers to problems in aquaculture has been introduced only recently, and again salmonid fish were the first to be studied (e.g., Estoup *et al.*, 1993). The extreme allelism of VNTR loci makes them ideal for identification and discrimination of stocks, especially in the case of species that display low levels of genetic variability in conventional markers, such as allozymes and mtDNA (Wright, 1993). Because VNTR scoring is PCR-based, thus requiring minute amounts of DNA from either fresh or preserved tissues, new opportunities have been arisen to address questions, that were very difficult to be approached in the past. It is now possible, for example, to evaluate the historical impact of stocking practices, by using archival samples, such as alcohol preserved fish or tissues. Also, early life-history stages, such as eggs and larvae have become now amenable to genetic analysis.

Microsatellite DNA primers have already been developed for the two most important species for mariculture in the Mediterranean basin. In 1993 three pairs of primers were developed for gilthead sea bream at the Institute of Marine Biology of Crete (IMBC) (Batargias and Zouros, 1993). These primers, plus an additional one, were used for the genetic characterization of the same Greek broodstock as the one mentioned above (Magoulas *et al.*, 1994, Batargias *et al.*, 1997). All four loci (SA1, SA26, SA32, SA41b, named according to the serial number of the clone from which the corresponding nucleotide sequence was determined) proved to be highly polymorphic, revealing 22, 16, 13 and 18 alleles respectively, in the 32 individuals comprising the stock. The average heterozygosity across all loci was approximately 0.95. These figures rank the gilthead sea bream microsatellites between most highly polymorphic loci ever found. This high level of polymorphism provides a very good resolution power for genetic tagging and parentage identification. At present, ten pairs of primers for single microsatellite loci have been developed at IMBC, in collaboration with the Institute of Molecular Biology and Biotechnology (Iraklio, Crete) (unpublished data).

Comparison of several wild and reared stocks of gilthead sea bream from four countries (Greece, Italy, Spain, Portugal), comprising approximately 450 individuals (5 samples of reared stocks and 4 samples of wild populations), is presently in progress in the context of an EU funded (AIR3) project. Preliminary results obtained by scoring the samples for loci SA26 and SA41b revealed 23 and 30 alleles respectively. For SA26, all samples (reared and wild) were found statistically different from each other in their allelic distributions (Fisher exact tests). For locus SA41b all reared samples were different to all wild samples and to each other, while all wild samples were not different to each other, with the exception of only a pair (Italy - Atlantic coast of Spain). However, it seems that the differences found, even if statistically significant, may not reflect substantial genetic differentiation, since differences of major magnitude, such as differences in the size range of alleles, between the stocks were not observed.

In 1995 the development of seven microsatellite markers in sea bass was reported (García de León *et al.*, 1995). All microsatellites were found highly polymorphic, displaying 0.86 heterozygosity and 7.6 alleles per locus in 6 breeders of a French stock. The Mendelian mode of inheritance and the feasibility for parentage identification were verified through analysis of the offsprings of crosses.

Monitoring population-level phenomena

The basic requirement of any culture programme is the existence of genetic variation. Therefore, an important aspect of cultured populations is their exposure to inbreeding and loss of genetic variation due to reduced effective population size. Inbreeding could be induced even in large stocks by behavioral, physiological and other factors (provided they have some degree of genetic determination). Random drift and loss of variability can occur if renewal of stocks is practiced using related individuals, if few individuals monopolize the sperm or egg pool or if the sex ratio becomes strongly biased, which is a possibility in a species with sequential sex reversal.

Allozyme assays have been very successful in detecting the genetic impact of culture. Studies have revealed retention of high enzyme heterozygosity levels in cultured rainbow trout, but there have been also cases of significant losses of allozyme variation (Ferguson, 1994, and references therein). Variation in mtDNA can be a more sensitive indicator of maternal genetic history than allozymes (Ferguson *et al.*, 1993). The higher sensitivity of mtDNA to phenomena such as genetic drift and founder effect make this marker ideal for monitoring the consequences of founding and propagation in aquaculture. Microsatellite DNA analysis has been recently used for such studies. In the context of the AIR3 project cited above, the evidence collected so far suggests that deviations from random mating in the reared stocks are small and that these stocks are not significantly less variable than wild populations. On the other hand, analysis of the F1 generation of a Greek broodstock (see below) revealed a 15% reduction in the number of alleles and a homozygosity increase of 1.5%.

Quantitative genetics studies

In traditional programs for genetic improvement in aquaculture several individuals of commercially important fish are pair-mated with one or more individuals of the opposite sex. When the offsprings reach marketable size, a large number of progeny of each cross are scored for the character under consideration and estimation of the heritability, as well as identification of the parents with the highest breeding value, are obtained from parent/offspring regression and/or correlation analysis of full or half sibs. Taking into account that a large number of pair-matings are needed for the estimates to be reliable, it is obvious that experiments of this kind can be very costly and impractical. Especially in the case of fish species, like the gilthead sea bream, in which pair-matings are impossible, the classical method is completely inapplicable, even if one could ignore the cost involved.

It seems that the development of molecular markers and especially microsatellites

have provided the solution to this problem, because it is now possible by using a small number of microsatellite loci, to give a unique genetic identity to each parent in a mass spawning and to identify its progeny. This, in turn, allows one to obtain correlations between progeny scores and parents. Herbinger *et al.* (1995) were the first to demonstrate the feasibility of determining pedigrees in a mixed family rainbow trout population, using a small number of microsatellite markers. Studies of this type are still not very common in the literature.

The first attempt to apply this approach to a marine species was undertaken successfully at the Institute of Marine Biology of Crete for gilthead sea bream (Batargias *et al.*, 1997). The previously mentioned 32 breeders, were put together for mass spawning, the eggs of a single-day spawning were collected, and the offsprings were reared in a common tank until the age of 6 months. At this point 150 individuals were removed at random, weighed and frozen. Another random sample of 150 offsprings was removed and treated the same way at the age of 10 months. Meanwhile, the sex of the breeders was determined by checking for presence or absence of sperm by the end of reproductive period.

Table 2. Number of offsprings per family in a mass mating of *Sparus aurata* (from Batargias *et al.*, 1997)

M \ F	2	4	12	18	26	28	29	30	31	Total
7	7		2	5	25		5	27	1	72
9	1			1						2
10	7	1		3	8		4	1	6	30
11	19									19
15	31	1	1	5				14		52
17	1		8	2			5		4	20
19	2						1		1	4
20	11						3		6	20
21				1						1
22			1				3			4
24					12		5		3	20
25							2			2
32	3		4	6	4	1		19	13	50
Total	82	2	16	23	49	1	28	61	34	296

Both samples of offspings were genotyped for the same 4 microsatellite loci, for which the parents were scored. Both parents of the offspring were unambiguously identified in the vast majority of individuals: 296 out of 300. Table 2 gives the 47 families that were detected for both age classes (some of these families were present in both age classes, but some others were not, with the result that 39

families were present for the 6- months class and 34 families for the 10-months class). It can also be seen that from the 32 breeders only 22 (9 females and 13 males) actually participated in the production of the progeny. Moreover, the contribution of these parents to the production of progeny was very unequal: the chi-square test rejected the null hypothesis of equal gametic contribution, both for "fathers" and "mothers".

To assess the parental impact on the growth of the progeny the non-parametric method of Kruskal - Wallis was used (Zar, 1984), because of unequal representation of parents in the offspring pool and because of lack of normality in the scores of size. For each parent a value, that was designated as "parental value", was estimated by:

$$\frac{\sum_{n}^{I}(W_x - \overline{W_p})}{n}$$

where W_x is the weight of offspring x, $\overline{W_p}$ is the mean weight of the offsprings of the other parent of the offspring, and n is the number of offsprings of the parent into consideration.

The results of the Kruskal - Wallis test are given graphically in Fig. 1. It can be seen that in both age-classes fathers form statistically different groups, while there is no such grouping of the mothers for the 6-months class, and only a minor grouping for the 10-months class. Statistical tests (Kendal's τ and Person's r) for correlation between the hierarchical order of the parents in the two age classes showed a significant coefficient in the case of fathers, but no significant correlation was found for the mothers. It seems then that there are significant differences between the male parents concerning the weight of their progeny, but such differences do not exist between female parents. In order to test for repeatability of the patterns observed, a similar protocol is being applied to the generation produced by the same breeders in another year.

Assessment of genetic manipulations

A variety of methods exist for genetic manipulation of fish, such as polyploidy and gynogenesis. Genetic markers are invaluable for confirming that the desired manipulations have been successful. For example, microsatellite loci are ideal for confirming the triploidy, because their high polymorphism makes it possible to detect triploid animals, by the observation of three-allele genotypes at certain microsatellite loci. This has been done in the case of induced triploidy in *Crassostrea gigas* oysters by using three microsatellite loci, in context of an EU funded (AIR1) project (unpublished data).

The detection of paternal inheritance in gynogenes is of critical importance and can most effectively be accomplished by using genetic markers. This has been done by allozyme markers in several cases, but VNTR loci, especially the single-locus ones, are very suitable for such assays (Ferguson, 1994). Highly polymorphic

genetic markers can also be used for the discrimination between meio- and mitogynes, by assessing the level of homozygosity, which is expected to be complete in the case of mitogynes.

Another application of genetic markers to aquaculture relates to the identification of genetic sex in monosex populations produced by sex reversal of females into males (masculinization) (e.g., Devlin *et al.*, 1991). Finally the assessment of integration, expression and germ line transmission of introduced gene in transgenic fish is dependent on nDNA-based techniques. Neither allozymes nor mtDNA markers can be used to this end (Ferguson, 1994 and references therein).

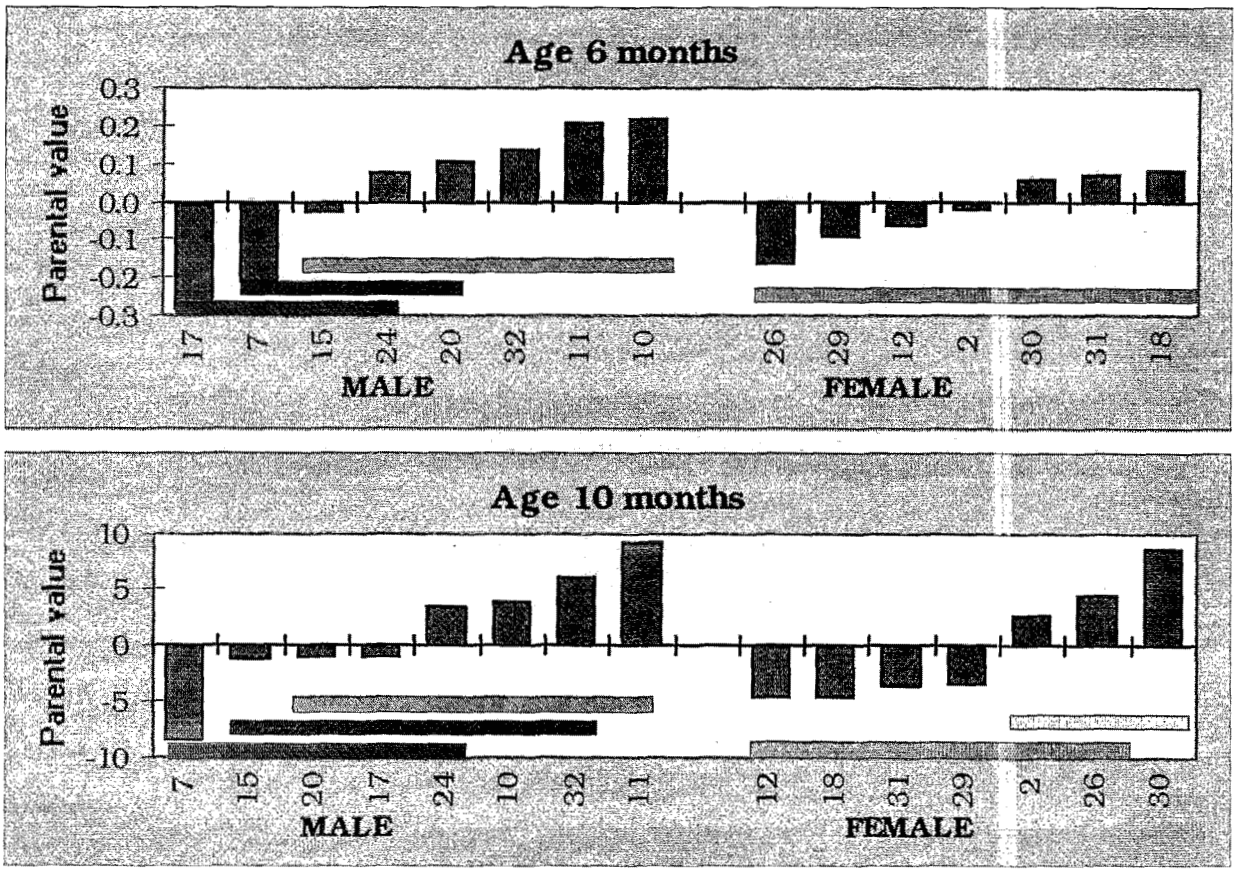

Fig. 1. Graphical presentation of "parental value" of the *Sparus aurata* breeders in a mass mating experiment. The hatched bars indicate the statistically different groups of parents (see text for details) (modified from Batargias *et al.*, 1997).

Concluding remarks - Future developments

Allozyme and mtDNA markers have provided valuable information for management in aquaculture. However, recent years witnessed the shifting of the main focus of interest from these markers to nDNA ones and especially to microsatellites. The introduction of microsatellites has the potential to revolutionize the use of molecular genetic markers in aquaculture. The reproducibility and the

ability for absolute size determination allow digital storage and comparisons of large number of individuals (O'Reilly and Wright, 1995). The pace of technological innovation is remarkable. Systems for automated detection and scoring of microsatellites are continuously growing in numbers, as do mathematical models for the analysis of microsatellite variation, a field that is presently in a stage of very active development. Non-radioactive scoring methods are already widely used. The introduction of the technique of multiplexing (amplification of multiple loci in the same reaction and running the products in the same gel lane) is a major technological advancement. It seems very likely that sample sizes unimaginable in the past will be processed routinely in the future. Nevertheless, one should always keep in mind that our power to resolve problems in the area of population genetics is always better served if alternative assays are used simultaneously.

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